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STUDIES ON THE PHYSIOLOGY, BIOCHEMISTRY, AND CYTOPATHOLOGY OF THE CORNEA IN RELATION TO INJURY BY MUSTARD GAS AND ALLIED TOXIC AGENTS

By Members of the Staff of the Wilmer Institute

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STUDIES ON THE PHYSIOLOGY, BIOCHEMISTRY, AND CYTOPATHOLOGY OF THE CORNEA IN RELATION TO INJURY BY MUSTARD GAS AND ALLIED TOXIC AGENTS

BY MEMBERS OF THE STAFF OF THE WILMER INSTITUTE

I. INTRODUCTION AND OUTLINE

JONAS S. FRIEDENWALD AND ALAN C. WOODS.

A study of the ocular injuries produced by chemical agents was begun at the Wilmer Institute in the summer of 1941. A few months later the Committee on the Treatment of Gas Casualties of the National Research Council requested that this work be greatly expanded and accelerated. A contract was negotiated between the Office of Scientific Research and Development and the Johns Hopkins University for the support of the expanded program in November 1941, and this support was continued until the end of 1945. The story of the arrangements under which work in this and related fields was carried out in a large number of laboratories has been told by Dr. Milton C. Winternitz (1), who acted as Chairman of the Committee on the Treatment of Gas Casualties and whose wise judgment and driving enthusiasm gave form and flexibility to the whole project.

In the first World War the incidence of casualties due to the ocular effects of mustard gas was extremely high, some ninety per cent of all gas casualties having ocular symptoms. For the most part these were relatively mild vapor burns which left no permanent damage to the eyes, but which prevented the individual from performing active duty for a few days to several weeks. Throughout the first part of World War II competent military authorities felt that there was considerable probability that gas warfare would be resorted to, and it was not until after the successful landing in Normandy that anxiety in this matter diminished. Research in this field was, therefore, under the pressure of imminent concern.

During World War I, research on the ocular injuries produced by war gases was largely limited to clinical and histopathological studies

on man and animals. Some laboratory experiments were performed seeking to establish a definitive treatment with various irrigations, ointments, etc., all of which were, however, found to be essentially inefficacious. In the work undertaken in the Wilmer Institute, it was realized from the outset that the chances were very small indeed of finding an effective treatment of mustard injuries of the eye by a continuation and expansion of the empirical methods of study used during World War I. Consequently, a program of study was developed on the broadest feasible base in the hope that, if we knew more about the mechanisms by which tissue damage results from toxic injury, some therapeutic possibilities might be disclosed.

It must be admitted at once that no cure for mustard injury was discovered. However, the search for the mechanism of mustard injury led us to study many aspects of normal corneal physiology that had not been previously explored. These phases of our study are reported in the series of papers which follow. Some parts of the investigation in respect to toxic agents other than mustard have been reported elsewhere (2–7).

OUTLINE OF THE INVESTIGATION

I. *Preliminary and Exploratory Studies.* As a first step it seemed desirable to discover whether there were any recognizable distinctions in the severity and type of clinical and histological reactions of the cornea to a variety of chemical agents of known reactivity. These studies were conducted by Dr. Wm. F. Hughes, Jr. It soon became apparent that some substances were well tolerated by the cornea when dropped on its surface but caused severe damage when injected into the tissue. Some useful information as to the permeability of the tissue may be gathered from this type of comparison. In order to study the intrinsic toxicity independent of penetrating power, we employed intracorneal injections in most of our tests. The results of this study are compiled in Appendix I.

It is possible to divide the injurious agents into two major groups, those that are damaging only in massive concentration and those that are damaging in very small doses. Among the former are acids and alkalis which cause injury by pH change or protein precipitation, hygroscopic organic solvents such as propylene glycol, hypertonic

solutions of inorganic salts, etc. Among the second group that are damaging in low concentrations are heavy metal ions, oxidizing agents including quinones, and alkylating agents. A possible common point of attack of this latter group are the sulfhydryl groups of the tissue. A ketobinder such as semicarbazide was tolerated by the tissues in considerably higher concentration than were the sulfhydryl attackers. The same is true of agents such as KCNO and formalin which react with amino groups.

While these results suggest that the war gases capable of damaging the cornea in very small doses may operate through an attack on the tissue sulfhydryl groups, the implication is by no means strong. In one sense these experiments were disappointing. No general distinctions could be found either in respect to the clinical or histological course of the lesion which could be related to the postulated mode of chemical attack of the injurious agent. Individual agents exhibited minor differences in the spreading of their effect, or the intensity of the purulent reaction which they elicited, but there were no systematic differences found between the effects of sulfhydryl binders such as arsenite, ketobinders such as semicarbazide, and amine binders such as formalin, or even between these groups and such unique agents as fluoride, quinine, cyanide, and the like. Even the effect of beta radiation, though remarkable for its long incubation period, is not grossly distinguishable from that of the various chemical agents. It is to be concluded that the clinically and histologically recognizable changes which follow chemical damage of the tissue are only remotely and indirectly connected with the initial chemical injury. Consequently, observations on these remotely connected events throw no light on the nature of the initial chemical injury.

This was perhaps to have been anticipated, for the clinical and histolgical reactions disclosed by routine study are, in the main, the consequences of cellular death. Once the cells are dead the reactions have a monotonous uniformity. This does not preclude the possibility that a study of the mode of death would be equally unilluminating, but such a study was beyond the scope of the routine examinations that were pursued in this phase of our investigation.

In spite of this disappointment these studies were not without value. They gave us a broadly based knowledge of the general course of

clinical reactions to tissue damage in the eye upon which we were able to construct a numerical index of the severity of the injury, an index which was to prove useful in the evaluation of therapeutic agents in later work. The method of applying this numerical index has been reported elsewhere (2). Moreover, knowledge was gained of a variety of agents that are surprisingly well tolerated by the ocular tissues. This knowledge was useful in the subsequent choice of possible therapeutic agents and vehicles. This aspect of the work was on a rather limited scale and could profitably be greatly expanded. Finally, a comparison of the effects of surface application with those of intracorneal injection furnishes a useful tool for the study of the permeability of the surface barrier, a subject which merits further extensive study.

II. *Clinical and Histological Studies on the Ocular Effects of Various War Gases.* Following these preliminary exploratory studies much more extensive and detailed studies of a similar kind were made by Drs. Hughes, Scholz, and Maumenee, using various war gases: mustard, the nitrogen mustards, lewisite, and one of the fluorophosphates. The studies by Hughes on lewisite have been reported elsewhere (5). Those by Maumenee and Scholz on mustard and the nitrogen mustards are presented in one of the succeeding papers (see page 121). Much of our effort in these studies was directed toward the production of a standard and reproducible lesion of submaximal severity for subsequent therapeutic tests.

Special reference should be made to the study by Dr. Scholz of the effects of di-isopropyl fluorophosphate which was found to be well tolerated by the ocular tissues, producing no local permanent damage even when administered locally in barely sublethal dosage. The analysis of the intense cholinergic effects produced by this agent in the eye is reported elsewhere (6, 7).

III. *Role of Secondary Infections.* The influence of secondary infections on the course of ocular injuries with war gases was studied by Drs. Maumenee, Guyton, and Burky. Using fairly severe injuries it was found that the suppression of infections by penicillin and sulfonamides resulted in a slight but definite reduction in the severity of the ocular reaction. Comparing these results with those of investigators in other laboratories, it would appear that the severity of

the secondary infection varies widely from one laboratory to another, and that more striking benefit from antibacterial agents is obtainable if the initial injury by the war gases is relatively mild. It was concluded that the results of studies on laboratory animals did not afford a valid basis for predicting how important secondary infections might be in humans, but that under proper precautions the antibacterial agents could be administered without danger of increasing the injury.

IV. *Antidotes.* 1) BAL (British Anti-Lewisite). The remarkable achievement of the British investigators, Peters, Stocken, and Thompson (8) in the synthesis of this compound and the demonstration of its anti-arsenical action placed the whole subject of the immediate treatment of war gas injuries in a new and different perspective. When this material first became available in this country, our laboratory, among others, was requested to study it. The matter appeared at the time to be one of great urgency and was pushed with the maximum possible speed. Using rabbits as the experimental animals, Dr. Hughes (5), who undertook this study, was able to work out the dosage of BAL tolerated by the eye, the effect of previous administration of lewisite on this tolerance, the therapeutic ratio of BAL, the influence of the time interval between exposure to lewisite and the administration of the antidote, the relative efficacy of the antidote against vapor and splash burns, etc. An effective ointment base was devised in which the antidote could be incorporated.[1] These results were rapidly extended to experiments on monkeys and, in relation to the tolerance of the eye for BAL, on human volunteers, and contributed a substantial base for the subsequent working out of the BAL eye ointment by others.

2) The Search for Antidotes against Mustard. The possibility of obtaining a completely effective detoxifying agent for mustard

[1] This ointment base was devised by Mr. Fuqua, pharmacist of the Johns Hopkins Hospital. Owing to security restrictions, it was not possible to tell him the nature of the material with which he was asked to work nor the purpose for which the ointment was being prepared. Nevertheless, with rare intuitive skill, Mr. Fuqua developed an effective ointment. The ointment base which he prepared has been frequently erroneously referred to as the "Friedenwald base." The writer wishes to take this occasion for making it clear that he had nothing whatever to do with the preparation of this ointment.

is rather poor, because no sustance has been found which will remove mustard from its combination with proteins without destroying the proteins. Consequently it can be hoped only that an antidote will neutralize the mustard which remains on the surface of the tissues plus that which has entered the tissues but has not yet reacted with them when the antidote is applied. It has been shown by others that in the skin approximately 10 minutes is required for a locally destructive dose of mustard to penetrate, while the half life of the mustard which has penetrated is of the order of two minutes. Consequently, within 5–10 minutes after exposure, surface decontamination is partially effective, but after 10 minutes almost all of the mustard which has penetrated has reacted with the tissues. In the eye the same argument holds a fortiori in respect to vapor burns, which in World War I produced most of the casualties. Consideration that mustard might be sprayed from airplanes during World War II enhanced the dangers of droplet contamination and made it desirable to explore the possibility of developing a penetrating decontaminant for the ocular tissues.

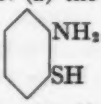
It was found that mustard penetrates the cornea more rapidly than the skin so that the time after exposure in which surface cleansing is still partially effective is very short (2–3 minutes). The rate of reaction of penetrated mustard with the cornea is of the same order of magnitude as its rate of reaction in the skin, the only favorable factor being that the cornea, because of the constant evaporation of the tears, is somewhat cooler than the skin. There is, consequently, a brief interval between 2 and 5 minutes after exposure during which a penetrating decontaminant might be expected to be beneficial.

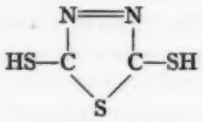
Within these limitations the agent to be sought for would be one with high reactivity for mustard, capable of penetrating into those regions in the tissues and in the cells reached by mustard, forming a non-toxic reaction product with mustard, and is itself non-toxic in therapeutically effective dosage. The problem is further complicated by the fact that the anterior corneal surface presents a relatively impermeable barrier of rather special character. It seemed wiser to avoid this special problem at the outset and to discover whether or not there were any substances which, if injected into the tissue simultaneously with mustard, would prevent its damaging effect.

To this end a screening test was devised in which mustard and the potential antidote were mixed in aqueous solution and immediately injected intradermally into rabbits. The same mixture was allowed to stand at room temperature for half an hour and then injected. Controls using mustard alone and the antidote alone were also injected. Initially lecithin was introduced into the injected mixture in the hope of imitating in this way the biphasic aqueous-lipoid arrangement of the tissues. Control experiments without lecithin showed that presence of this substance did not alter the test. Substances which effectively protected the tissue against mustard in the presence of lecithin also did so in its absence. Substances which were ineffective with lecithin were ineffective without it. Nevertheless, the presence of lecithin in the mixture was of considerable practical advantage, for the mustard in lecithin aqueous emulsion maintains its potency much longer than in the absence of lecithin. Consequently, the time interval between preparing the mixture and its injection is less critical when lecithin is included. Substances insoluble or poorly soluble in water were dissolved when possible in ethyl alcohol and then introduced into the lecithin mustard water emulsion in such quantity that the total concentration of alcohol was non-irritating. Later, dimethyl formamide was found to be a satisfactory vehicle for some substances insoluble in both water and alcohol. Substances insoluble in water or ethyl alcohol were dissolved when possible in dimethyl formamide and then introduced into the lecithin mustard water emulsion in such quantity that the final concentration of dimethyl formamide was non-irritating.

Several hundred substances of many chemical types were tested in this manner.[2] Three groups were found which contained some

[2] Some of these materials were available commercially. Others were supplied by Dr. Hellerman of the Department of Physiological Chemistry of the Johns Hopkins Medical School. A considerable number of biochemical substances were contributed by Merck and Co. Dr. Bergmann of the Rockefeller Institute contributed some materials especially prepared as possible antidotes for mustard. By far, the most important contribution of substances was supplied by the Chemical Department, Experimental Station of the duPont de Nemours Co., under a contract arranged by the 9-1 Division of the NDRC for this purpose. The cooperation and suggestions of Dr. Lazier and later of Dr. Howk who conducted the synthetic work at the duPont Laboratories, and of Dr. Marvel and later of Dr. Adkins as chairman of the 9-1 Committee are deeply appreciated by us.

effective members: (a) the dithio-carbamates $R_2NCSSNa$, (b) ortho-amino-thiophenol  and certain of its derivative and analogues, (c) 2,5 dimercapto-thio-diazole.

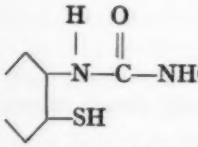


(a) A considerable number of dithiocarbamates were tested on rabbits' eyes following droplet contaminaton with mustard and nitrogen mustard. The lower members of the series, especially diethyl and diethanol dithiocarbamates proved the most effective and, under optimal conditions achieved close to the theoretically maximum therapeutic effect. Several disadvantages were found which made these substances unsuitable for supply to combat troops. They were found to be effective only when administered in concentrated aqueous solutions and the problem of packaging such solutions presented enormous difficulties. Salves and jellies were not found effective. Owing to the high hypertonicity of the effective solutions they caused marked immediate blepharospasm and lachrymation when administered to an unanaesthetized eye and achieved their maximal therapeutic benefit only when administered under general anaesthesia, consequently self treatment with them would be very difficult if not impossible. Finally, these substances could not be combined with BAL in the same preparation because of their mutually exclusive pH stability zones. It was not thought feasible to supply combat troops with two separate antiwar-gas eye preparations, nor to abandon BAL, which itself has some protective value against mustard in the eye, for another preparation which was only slightly better against mustard and had no value against arsenical agents. Nevertheless, the possible use of dithiocarbamate solutions for first aid in mustard factories was recommended.

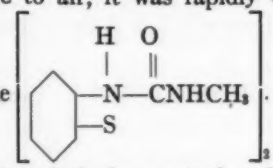
(b) Ortho-amino-thiophenol was the most effective substance found in the screening tests, in terms of the minimal molar ratio of

antidote to mustard that was protective. This substance is, however, quite toxic and its therapeutic index (minimal effective dose divided by minimal toxic dose) was very small. No mode of administering this substance to the eye effective after droplet contamination with mustard was found. A derivative of this compound, 3 mercapto 4 amino benzoic acid, and also its ethyl esther were found capable of some therapeutic benefit. These are, however, quite toxic and could be used only in low concentrations. Oxidation of these compounds and subsequent reduction by sulfhydryl containing substances resulted in an increase in their toxicity. It seemed possible that in such an experiment derivatives of the original compound were being formed which had an increased tendency toward quinonoid oxidation and that a similar course might explain the high toxicity of the original material in the tissue. Following the line of argument plans were formulated for the study of various N substituted derivatives of ortho-amino thiophenol.

One such compound (ortho-mercapto-phenyl-methyl-urea)



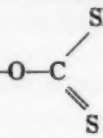
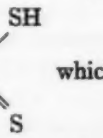
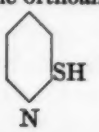
was prepared by the duPont laboratory. It proved to be so intensely auto-oxidizable that, in solution on exposure to air, it was rapidly converted into the corresponding di-sulfide



. Skin tests with the disulfide showed it to be relatively non-toxic. Solutions of the disulfide were reduced by H_2S and used with mustard in lecithin emulsion in the screening skin test. The compound was found to be about as effective against mustard as was 3 mercapto 4 amino-benzoic acid and also about as toxic as this previously tested substance. The nature of this toxicity was not further explored.

(c) The compound 2,5 dimercapto-thiodiazole was itself extremely

toxic and was not further explored since no possibility suggested itself as to how non-toxic derivatives might be produced.

We regret very much being compelled to abandon the search for an anti-mustard antidote with the job neither satisfactorily accomplished nor proven impossible of solution. An analysis of our data can yield only tentative conclusions but is offered here in case it should be found necessary to reopen this problem in the future. All of the compounds which were found effective in our screening test contain sulfhydryl groups whose acid dissociation is below pH 7. Consequently, they all presumably have quite high competition factors for mustard. But there are many other substances with equal or higher competition factors, for instance thiophosphates, which were found ineffective on the screening test. The presence of a nitrogen atom in close proximity to the SH group seems necessary for their effect. The xanthates  and the monosubstituted thiocarbamates  which are chemically closely related to the dithiocarbamates proved ineffective. The same is true of thiophenol and a number of its derivatives which lack the orthoamino group. On the other hand alpha-mercapto-pyridine  was slightly protective. When we first noted this relationship we suggested that the effective compounds might derive their efficacy from their capacity to form internal salts, but a subsequent study of the dissociation constants of the potential basic groups revealed them to be such weak bases that they could furnish only infinitesimal concentrations of dipole-ions at physiologic pH. A possible alternative explanation is that these compounds may exist in part as chelate structures. Such uncharged molecular species might be able to

penetrate into intracellular regions inaccessible to the anions of simple sulfhydryl acids. No studies have been made by which this hypothesis could be tested.

V. *Primary Site of Mustard Reaction.* With threshold doses of mustard, the only change in the corneal tissue that we have been able to recognize is a transitory inhibition of mitotic activity in the corneal epithelium. It would be of interest, therefore, to know whether the primary site of reaction of mustard with the cell is in the cytoplasm or in the nucleus. The lipoid solubility of mustard and its unreactive nature in the absence of water suggests that the aqueous phase of the tissues immediately adjacent to lipoidal structures might be the site of greatest reactivity of mustard.

(1) The electrical resistance of the cornea is not immediately affected by exposure to mustard. Some hours after exposure the resistance declines moderately (about 25%), and when the epithelium sloughs off it declines to zero. It may be concluded that the lipoidal phase of the cell membrane is not disrupted as the immediate result of the reaction of mustard with the tissue (see page 102).

(2) The nuclei of the corneal stroma cells show increased fragility after exposure to mustard but this is not an immediate result of exposure to mustard (see page 161).

(3) None the less it is evident that the corneal epithelial cells do not behave toward mustard as if they were homogeneous solutions. These cells in the beef eye contain considerable amounts of glutathione. If the corneal epithelium is scraped off from beef eyes, suspended in a small volume of salt solution and an amount of mustard added equimolar to the content of glutathione, no measurable decrease in the titratable glutathione is observed (experimental error 5%). If, however, a similar preparation of epithelial cells is first ground with broken glass and then the same amount of mustard added to the resulting emulsion, about 50% of the previously titrable glutathione disappears. It is evident that in the intact cell glutathione is relatively inaccessible to the mustard (see page 102).

(4) After very heavy exposures to mustard vapor there is an increased eosinophilic staining of the corneal tissues. This increased capacity to bind acid dyes is apparently due to the formation of some new basic groups in the tissue as the result of its reaction with mustard.

A possible explanation of this phenomenon is to be found in the tendency of mustard to form sulphonium compounds in certain reactions. After very heavy exposures the increased eosinophilia is uniformly distributed within the corneal epithelial cells, but as the dosage is reduced the regions about the nuclear membrane and the basement membrane appear to be the last to lose their enhanced eosinophilia. These results were obtained at the very limit of the sensitivity of the method used to observe them and are open to grave question. Nevertheless, they suggest that two of the regions specially susceptible to mustard attack may be in or adjacent to the nuclear membrane and the basement membrane (see page 102).

VI. *Nuclear Changes Produced by Mustard.* In the search for a field of research which might diminish the enormous gap in our knowledge between biochemical studies and those of clinical and histological pathology, we have investigated some of the nuclear disturbances which follow exposure to mustard. These studies were made by Dr. Friedenwald with the aid of Dr. Buschke.[3] The results of these studies are reported in detail in separate articles below (see pages 148, 161, 178).

(1) Inhibition of Mitosis. Inhibition of mitosis occurs after exposure to very small doses of mustard and allied agents, and represents the most sensitive index, so far discovered, of threshold effects produced by these agents. The inhibition comes on slowly and can be made to last for as long as a week without being associated with

[3] Friedenwald and Buschke had been engaged for some time in the study of mitotic and wound healing activities in the corneal epithelium. When it was decided to apply to the mustard problem the technique gained in this field arrangements were made to apply for the clearance of Dr. Buschke so that he could become a member of the group under OSRD contract. All concerned approved this step, but when application for clearance had been prepared it was learned that Dr. Buschke's father and mother had been taken into custody by the German government and placed in a concentration camp. Since the lists of those engaged in scientific work for the OSRD might well be available to German espionage it was decided that the inclusion of Dr. Buschke in our project might place unnecessary risks upon the lives of his parents. Consequently, Dr. Buschke agreed to work as a voluntary assistant on this problem, and assisted in the studies with great devotion in spite of being excluded from all knowledge of the nature of the agents used in the experiments and of all other confidential and secret information. His contribution to the work has been of very great value.

any signs of irritation or tissue destruction. No evidence is available with which to decide whether the primary damage responsible for mitosis inhibition is due to a reaction of mustard with nuclear or cytoplasmic components. The healing of small wounds by migratory action of the cells is not disturbed during the period of mitosis inhibition. Recovery from the inhibition is associated with a full return to normal. The inhibition occurs at levels of dosage insufficient to produce any of the metabolic disturbances which we have so far discovered.

(2) Nuclear Fragmentation. With doses of mustard somewhat greater than the minimum required to inhibit mitosis, but at thresholds still below the level of producing necrotizing lesions, some cells in the basal layer of the corneal epithelium show a form of karyorrhexis which we have called nuclear fragmentation. This peculiar mode of cellular death has been subjected to extensive study.

Under ordinary experimental conditions only a small number of the cells in the basal layer of the corneal epithelium are susceptible to this type of injury, but after various pretreatments the number of susceptible cells can be greatly increased. It is concluded that susceptibility to this type of injury is associated with some particular physiologic state of these cells though there are other tissues in which the cells are either uniformly susceptible or uniformly refractory to this type of injury. Evidence has been obtained which suggests that the susceptible state in the corneal epithelium is connected with the mitotic cycle, and that it may provisionally be identified as the pre-mitotic state, while the process of nuclear fragmentation itself may be provisionally identified as a pathological and incomplete form of mitosis. It thus appears that mustard and related substances can furnish important experimental tools for the study of mitosis. In this respect they deserve to take a place along with ultraviolet light and X-ray. Severe nuclear fragmentation, like mitosis inhibition, occurs at a dosage level which still fails to produce the metabolic disturbance noted below.

(3) Nuclear Fragility. The mode of death of the cells of the corneal stroma after exposure to mustard differs radically from the mode of death of the epithelial cells. Nuclear fragmentation does not occur in the corneal corpuscles. On the contrary after moderately severe

exposures the nuclei of these cells first swell and then burst. A study of this phenomenon showed that the swelling of the nuclei of the corneal corpuscles was a consequence of the imbibition of water by the cornea following damage by mustard to the corneal endothelium, and that similar swelling of the nuclei of the corneal corpuscles occurs after many types of damage to the endothelium. However, in corneal edema produced by mechanical destruction of the endothelium, the nuclei though swollen do not burst. The specific effect of mustard on these cells is, therefore, an increase in the nuclear fragility. The increased fragility of the nuclei appears only after some hours of incubation following exposure to mustard. Following massive exposures, cells of all types undergo pycnosis.

The phenomena of nuclear fragmentation and nuclear fragility develop not only in vivo but also in enucleated eyes maintained supravitally in an incubator. It is possible, therefore, to vary widely the experimental conditions both before and after exposure to the toxic agent. Experiments of this type have thrown some light on factors favorable and unfavorable to the accumulation of cells in the susceptible state and also on factors favorable and unfavorable to the development of the lesions. In the past, studies of this sort have been made by others on tissue cultures or on monocellular organisms. The cornea appears to be an extremely suitable organ for extension of the techniques of cytopathology from the field of single cells into that of organized structures. A further extension in the same direction is exemplified in the section which follows.

VII. *Cohesion of the Corneal Tissues.* One of the early pathological results of mustard injury is a loosening of the corneal epithelium. This can be seen both in the intact animal and in the isolated incubated cornea. Studies on this phenomenon were undertaken by Dr. Herrmann and are reported in detail below (see pages 182, 208, 213). The cohesive surface appears to be a protein-lipoid multilayer since it is disrupted both by trypsin and by such substances as butyl and amyl alcohol and some other detergents. Energy may be necessary for the normal maintenance of cohesion, for while cyanide and anoxia do not cause a loosening of the tissue, iodoacetate and fluoride do cause such a loosening. Histamine and also freezing of the tissue with subsequent incubation also lead to a loosening of the epithelium. It

is likely that this phenomenon is closely related to that of vesication in the skin.

Exposure to mustard causes no immediate loss of cohesion, but on subsequent incubation the epithelium becomes loosened. The loosening of the epithelium is not a consequence of the death of the epithelial cells, for small wounds in the epithelium heal normally even when the tissue has become loosened. The loosening of the epithelium does not occur on anaerobic incubation, and some recovery from this toxic effect of mustard is achieved by the tissue during anaerobiosis. It is evident that the pathologic process caused by mustard which leads to the loosening entails an oxidative step. Reduced temperature also slows down or prevents the development of the loosening, and some recovery from the injury takes place during incubation at the lower temperature.

Nuclear fragmentation is also inhibited by anoxia and by lowered temperature, but the temperature coefficients of the two pathological processes are not identical. Under proper experimental conditions either loosening or nuclear fragmentation can be produced alone. This rules out the possiblity that the loosening is the result of the accumulation in the tissue of toxic products derived from the autolysis of cells that are undergoing nuclear fragmentation.

VIII. *Studies on Corneal Metabolism.* Work in this field was conducted by Dr. Herrmann. In principle there are three modes of studying the effect of a poison on tissue metabolism. In the first place one may isolate various enzymes from the tissue and study their sensitivity to inactivation by the toxic agent. This mode of study has been widely pursued by others and has added much to our knowledge of the reaction of mustard with proteins in general, but has thrown little light on any possibly crucial enzymes upon the inhibition of which the pathological effects of mustard injury may be supposed to depend. We have not undertaken work in this field, since it was well covered by others. Furthermore there are some apriori objections to this mode of attack in relation to the specific objectives of our study. The known enzymes constitute only a fraction, possibly only a small fraction, of all those necessary for cellular activity. Even if an enzyme exquisitely sensitive to inhibition by mustard could be found the question would still remain open as to whether

this enzyme was accessible to mustard in the intact tissue (cf. the inaccessibility of glutathione to mustard in the corneal epithelium). Finally, it is possible that enzyme systems may be inactivated through structural changes or losses of intermediate factors without injury to the enzymes themselves.

A second mode of study, which has also been pursued by others, consists in exposing the tissues to mustard and then testing whether particular enzymes can be isolated in normal or diminished activities. This method has yielded some fruitful results in the hands of a number of investigators. It is free from the second objection mentioned above, but not from the other two objections. Furthermore, diminished extractability and diminished activity need not be identically related. Finally, work of this type was a natural corollary to that of the first type and was being actively covered by the efforts of others.

A third method of approach, the one we have followed, has been to study the utilization by the normal and poisoned tissue of various primary and intermediate metabolites. Once the interruption of particular metabolic activities could be demonstrated, the precise locus of the injury to enzymes, structure, or intermediate factor, could, we hoped, be attacked with more confidence. Work in this field was necessarily slow. At the outset almost nothing was known about the normal metabolism of the cornea, and only those metabolites could be tested for which satisfactory micro-estimation methods existed or could be elaborated. On the other hand, the cornea seemed an especially favorable tissue on which experiments of this type might be performed. Its avascular character renders its supravital maintenance extremely simple. Its anatomical simplicity made the separation of epithelium from connective tissue readily feasible, and hence made possible the study of the metabolic effect of poisoning on the separate tissue components.

The results of these studies are reported in detail below (see pages 225, 251, 260, 273, 287, 295). There is no evidence that the overall supply of available energy in the mustard poisoned tissue is defective because the conversion of glycogen to lactate proceeds at a normal rate. There is, however, some diminution of oxygen uptake. Moreover, as noted above, some abnormal utilization of oxidative energy occurs in the development of nuclear fragmentation and of epithelial loosening. It has not

been possible, so far, to specify precisely in what respects the oxidative processes are abnormal. The available evidence is discussed in detail in the papers by Dr. Herrmann, and in the final summary (page 326).

A prominent feature of corneal metabolism revealed in this work is the metabolic interaction between epithelium and stroma. The epithelium was found to have a carbohydrate metabolizing system similar to that of many other tissues involving a cyanid sensitive oxygen acceptor, hexose phosphorylation and cleavage. The stroma has no measurable oxygen uptake but can consume glucose at a rate per cell twice that of the epithelium. Part of the glucose utilized can be recovered as lactate but the tissue has no power of utilizing lactate. In the isolated cornea the lactate produced by the stroma is consumed by the epithelium, in spite of the fact that the concentration of lactate is generally higher in the epithelium than in the stroma. Conclusive proof that lactate is actively transferred from stroma to epithelium is lacking, but the utilization of the stroma lactate by the epithelium is completely inhibited by mustard poisoning. Similar results were obtained with serine which is also utilized by the epithelium but not by the stroma.

It is to be emphasized that the interference by mustard in the intercellular metabolism is not all embracing. Pyruvate injected into the stroma is utilized at a normal rate after mustard poisoning. Glucose utilization is also not inhibited. Nor is interference with the intercellular metabolism the sole result of mustard injury. Quite obviously, the tissues even if mechanically separated from one another are susceptible to injury from mustard. We wish merely to point out that one of the aspects of mustard injury is a disturbance in the intercellular metabolism of the cornea and that mustard furnishes a powerful tool for the study of this hitherto poorly explored field of tissue metabolism.

In one large field our attempt to study corneal metabolism met with complete failure. It was hoped that by using nitrogenous metabolites containing N^{15} we would be able to study the course of synthetic activities in the tissue. A mass spectrograph was placed at our disposal by the Department of Chemistry of the Johns Hopkins University for this purpose and considerable funds were used in an effort to adapt this instrument to our purposes. Unfortunately,

the instrument was designed for high resolution and yielded an ionic stream of extremely low amperage. In spite of much effort it was found impossible to adapt this instrument for quantitative estimation of N^{15}/N^{14} ratio in samples, and the effort had to be abandoned. The difficulties here were wholly technical and there is no reason why work in this field will not some day yield important results. Indeed, preliminary runs analyzed for us by Dr. Rittenburg of Columbia University showed that the nitrogen turnover of a single isolated beef cornea yielded measurable amounts of protein synthesis.

PERSONNEL

Mention has been made above of the special contributions of several of the individuals in our group, but this by no means suffices to acknowledge the share of various others nor adequately to record the uniform enthusiasm and devotion of the whole group. The appended table 1 lists all of the members and the main field of activity of each.

COOPERATION WITH OTHER GROUPS

1) Department of Physiological Chemistry, Johns Hopkins Medical School. In undertaking work in the field of chemical warfare with which we had no previous acquaintance, need was felt for help from more experienced colleagues. Dr. W. M. Clark, Professor of Physiological Chemistry at the Johns Hopkins Medical School, generously placed the cooperation of his staff and the facilities of his department at our disposal. In order to protect the patients in the Wilmer Institute from risk, stock supplies of toxic agents were stored in the laboratory of Physiological Chemistry under the care of Dr. Curt C. Porter. Dr. Leslie Hellerman and Dr. Barnett Cohen gave most generously of their time and thought to our problems. Dr. Hellerman accepted the position of consultant for our group and on several occasions performed a number of titrations and preparations for us. In this he was assisted at one period by Mr. Presta, and later by Dr. M. R. Bovarnick.

2) Other Ophthalmological Groups. During the course of the work, conferences were held from time to time with the other ophthalmic groups working in this field. These conferences were arranged by the Committee for the Treatment of Gas Casualties and proved

extremely useful in dividing up the domain of study, in arranging mutual checks in respect to special experiments, in occasional direct collaboration, and particularly in formulating the conclusions of the various studies in terms of draft directives for use by the armed forces.

TABLE 1

NAME	FULL OR PART TIME	LENGTH OF SERVICE	TITLE	CHIEF FIELD OF ACTIVITY
1. Dr. Alan C. Woods		11/1/41–12/31/45	Responsible investigator	In charge of administration.
2. Dr. Jonas S. Friedenwald		11/1/41–12/31/45	Responsible investigator	In charge of research.
3. Dr. Roy O. Scholz	Full	4/1/42–7/1/45	Ophthalmologist	Pathology and treatment of mustard burns.
4. Dr. Wm. F. Hughes, Jr.	Full ½	4/1/42–7/1/43 7/1/44–12/31/45	Ophthalmologist	Pathology and treatment of Lewisite burns.
5. Dr. Albert Snell, Jr.	½	9/1/43–10/1/44	Ophthalmologist	Screening test for mustard antidotes.
6. Dr. Alfred E. Maumenee	Full	4/1/43–5/1/44	Ophthalmologist	Pathology and treatment of nitrogen mustard.
7. Dr. Jack S. Guyton	Part	1/1/43–1/1/44	Ophthalmologist	Secondary infections.
8. Dr. Wilhelm Buschke	Part	1/1/43–12/31/45	Ophthalmologist	Effect of mustard on the mitotic activity and nuclear fragmentation in the corneal epithelium.
9. Dr. Heinz Herrmann	Full	7/1/42–12/31/45	Chemist	Effect of mustard on corneal metabolism; loosening of the corneal pithelium.
10. Dr. Samuel A. Talbot	Half	7/1/43–7/1/45	Physicist	Mass spectrograph for N^{15} estimations.
11. Dr. Lealie Hellerman	Part	7/1/42–12/31/45	Chemical consultant	Consultant.
12. Mr. Ulric A. Presta	Part	7/1/43–10/1/43	Chemist	Assistant to Dr. Hellerman.
13. Dr. Marianna R. Bovarinch	Part	10/1/43–1/1/44	Chemist	Assistant to Dr. Hellerman.
14. Dr. Earl L. Burky	Part	4/1/42–4/1/43	Bacteriologist	Secondary infections.
15. Fay H. Hickman	Full	4/1/42–10/1/44	Technician	Assistant to Dr. Herrmann.
16. Jane E. Crowell	Full	6/15/43–7/1/45	Technician	Assistant to Dr. Friedenwald and Dr. Buschke.
17. Dorothy Breeskin	Full	7/1/45–12/31/45	Technician	Assistant to Dr. Friedenwald and Dr. Buschke.
18. Sylvia G. Moses	Full	8/1/43–7/1/45	Technician	Assistant to Dr. Herrmann.
19. Grace Futcher	Half	7/1/43–12/1/43	Technician	Assistant to Dr. Talbot.
20. Nathalie Weisgall	Half	11/1/43–7/1/45	Technician	Assistant to Dr. Talbot.

The exchange of ideas between the various groups proved mutually stimulating.

3) Committee on Physiological Mechanisms of NDRC. The close relation between our work and that of the Committee on Physiological Mechanism of the NDRC was recognized early and arrange-

ments for mutual exchange worked out. The Committee invited representatives of our group to attend the regular scientific meetings of its contractors and to report, on occasion, phases of our work that were relevant to their interests. These meetings and the informal exchanges that surrounded them were extremely useful both in respect to the exchange of ideas and in arrangements for complementary and collaborative undertakings.

4) Research Laboratory of duPont. Shortly after the development of our screening test for prospective mustard antidotes, it became apparent that considerable synthetic work in the preparation of compounds for testing would be desirable. Arrangements were made by Dr. Winternitz and Dr. Marvel for a contract with the Chemical Department, Experimental Station of the E. I. duPont de Nemours Co. through the 9-1 Division on Organic Chemistry of NDRC. The work at duPont was conducted first by Dr. Lazier and later by Dr. Howk. Almost 100 compounds were prepared. Both Dr. Lazier and Dr. Howk and their associates gave most thoughtful consideration to the problem and made numerous suggestions which became incorporated in the work. Dr. Marvel and Dr. Adkins, his successor as chairman of the 9-1 Division, were continuously helpful.

5) Edgewood. Cooperation was arranged with the staff at Edgewood Arsenal on a number of field tests and surveys of exposed personnel. In addition, Major Laughlin, Chief of the Division of Ophthalmology of the Medical Research Laboratory at Edgewood, was throughout the period of joint work continuously in close touch with our laboratory, and on a number of occasions performed checking experiments in relation to some of our studies.

SUMMARY AND DISCUSSION

The coordinated study which has been outlined above fell far short of its goal. No efficacious method for the treatment or prevention of mustard injury of the eye was achieved as the result of our work. On the basic problems of the physiology, biochemistry, toxicology, and cytopathology of the cornea we feel that we have done little more than scratch the surface. For a deeper penetration into these fields much more time and many more techniques will no doubt be required. Our reasons for publishing the relatively unconnected aspects of this study

in a single issue and for presenting the outline of the work in this introductory chapter, are that we hoped in this way to point out the interrelations, however tenuous, between the different parts of the investigation. The mutual fertilization involved in this coordinated effort was the factor chiefly responsible in enabling us to open up several new fields of experimental study.

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II. PRIMARY REACTION OF MUSTARD WITH THE CORNEAL EPITHELIUM*

JONAS S. FRIEDENWALD, ROY O. SCHOLZ, ALBERT SNELL, JR., AND SYLVIA G. MOSES

Studies have been undertaken by a number of investigators to determine tissue components that react with mustard. There is general agreement that the tissue proteins are chiefly concerned in these reactions and many enzymes have been shown to be inactivated by reaction with mustard. Various reactive groups normally present in proteins, particularly sulfhydryl, amino, and carboxyl groups have been shown capable of reacting with mustard, and in homogenous solutions sulfhydryl compounds show the highest competition factors among the biochemical substances. Presumably, therefore, a part of the mustard bound by tissue is bound by sulfhydryl side chains of proteins. Studies with mustard synthesized from radioactive sulfur have in the hands of Henriquez (1) revealed much useful information as to the proportion of reacting mustard which is bound by the skin, the rate at which penetration and binding takes place, and location of bound mustard in the affected tissue. Similar studies by Hamilton and Axelrod (2) have added much to our knowledge of similar factors in respect to the cornea. The resolving power of radio histograms, however, is inadequate to reveal individual cells. The following investigations were undertaken with the aim of throwing light on the primary reaction of the mustard with the tissue. It cannot be claimed that these studies have given an answer to this question. The results, however, are of some interest and some of the methods used may have potential applicability to other cytochemical fields.

I. AMOUNT OF MUSTARD IN CORNEA

In the experiments to be reported in this and in subsequent papers eyes were exposed to mustard vapor at room temperature (22–24°C.) generally in one of two ways. In some experiments the eyes were placed over the mouth of a cylindrical weighing bottle 5 cms deep,

*The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

the bottom of the bottle being covered by a layer of liquid mustard. Dosage was varied by varying the time of exposure. This technique was, in general, used for exposure of enucleated beef eyes. In other experiments the eyes were placed over the mouth of a similar cylindrical vessel to which a small bore glass tube was attached as a side arm (see Fig. 1). A slender wooden stick (swab stick) was inserted through the tube and a small piece of filter paper inserted in the end of the stick within the vessel. Mustard was placed not only on the floor of the vessel but also on the filter paper. By rotating the stick a fanning action of the filter paper could be produced and the air in the chamber kept saturated with mustard vapor. The vessel was pro-

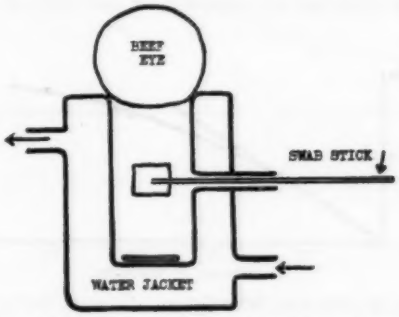

FIG. 1. CHAMBER FOR EXPOSURE OF CORNEAS TO SATURATED MUSTARD VAPOR

vided with a double wall through which water could be circulated to keep the temperature constant. Dosage could be controlled either by varying the time or the temperature of exposure.

It was desirable to find out how much mustard entered the cornea per minute with these types of exposure. Experiments were performed in which circular buttons of beef cornea, of known area, cut with a cork borer were placed on the under surface of the glass stopper or shallow vessels (2.0 cm. deep) of the first type (i.e. without the fan). After exposures of varying periods the buttons were removed and immediately immersed in isopropyl alcohol for 20 minutes, during which time they were ground and pressed intermittently. Further extraction did not increase the recovery significantly. At the conclusion of the period of extraction, a 2.5 ml. portion of the isopropyl

alcohol extract was used for the colorimetric determination of its mustard content by a procedure not yet released for publication. The lower limit of sensitivity of the method is about 2 micrograms in 5 ml. The results of these titrations are charted in Fig. 2. Extrapolating the curve to zero time and drawing the tangent to the curve at the origin one obtains an estimate of 3.3 micrograms uptake of mustard per square centimeter per minute.

In a second series of experiments the corneal buttons after exposure to mustard vapor for 20 minutes were transferred to another similar but empty vessel and allowed to remain in this vessel at room temperature for varying periods before being immersed in isopropyl alcohol. These titrations afforded an estimate of the rate at which mustard

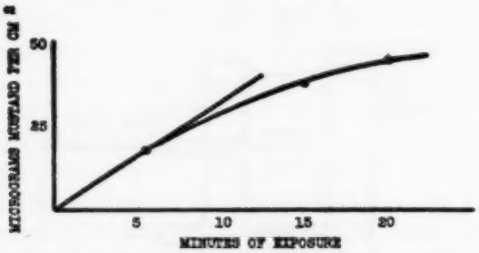

FIG. 2. UPTAKE OF MUSTARD BY BEEF CORNEA EXPOSED IN 2 CM. DEEP CHAMBER

reacts with the tissue. The results are shown in Fig. 3. Extrapolating this curve back to zero time and drawing the tangent to the curve at its beginning one reaches the conclusion that about 8% of the mustard in the cornea disappears per minute. Referring back to Fig. 2, it is seen that the concentration of mustard in the cornea on continuous exposure to vapor under these experimental conditions tends toward a maximal level of about 50 micrograms per square centimeter. At this steady state the rate of uptake should equal the rate of disappearance. The rate of disappearance is 8% per minute or 4 micrograms per cm^2 per minute. These two methods therefore give reasonably good agreement.

Similar experiments with deeper vessels (5 cm. deep) yielded an uptake of 0.6 micrograms per cm^2 per minute, while experiments with vessels with the fan (saturated mustard vapor) gave an uptake of 4.0

micrograms per cm² per minute, and exposure to liquid mustard showed an uptake of about 8 micrograms per cm² per minute. All of these experiments were performed at 22°C.

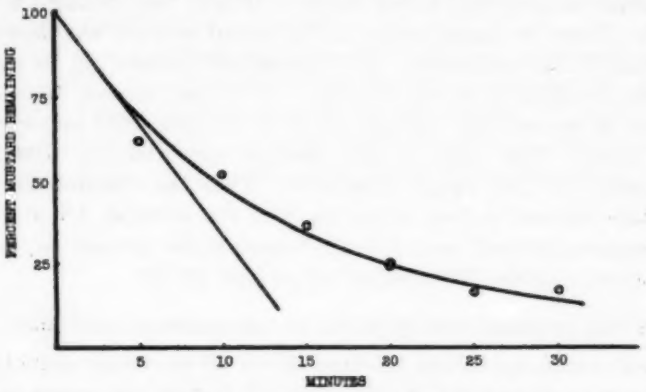

FIG. 3. DISAPPEARANCE OF MUSTARD FROM CORNEA AFTER EXPOSURE TO VAPOR

TABLE I

Relation of Mustard Uptake to Effects Observed

MICROGRAMS MUSTARD PER CM²	RABBIT	RAT	BEEF
10	Cornea perforates Permanent scarring Loosening of epithelium	Cornea perforates	
1		Permanent scarring Loosening of epithelium	Threshold for loosening of epithelium and metabolic effects
0.1		Threshold for nuclear fragmentation	
0.01		Threshold for mitosis inhibition	

Using these figures together with experimental results to be reported below, it is possible to estimate the amount of mustard required to produce various corneal symptoms. The results are shown in Table I.

These data may be elaborated further with the aid of the following considerations. The studies (2) have shown that only about 15% of the mustard which reacts in the tissue becomes bound, presumably

by reacting with fixed elements of the tissue. The remainder is extractable, presumably in the form of thiodiglycol, and hence presumably having reacted with tissue water. Thus the effective portion of the absorbed mustard may be estimated as 15% of that indicated in the table. Since the major portion of the bound mustard was shown to be fixed in the epithelium, the following calculations can be made. At the threshold of mitosis inhibition for the rat's cornea, .01 micrograms of mustard per cm^2 are required. Of this .0015 micrograms are bound. The rat's cornea contains approximately 6,000,000 epithelial cells per square centimeter. Thus the effective dose to produce minimal mitosis inhibition is of the order of 2.5×10^{-10} micrograms per cell, or 1.5×10^{-18} gram moles per cell, or, using Avogadro's number 10^6 molecules of mustard per cell.

II. GLUTATHIONE AND MUSTARD IN THE CORNEAL EPITHELIUM

Beef corneal epithelium has been shown (3) to contain quite large amounts of glutathione, roughly 150 micrograms per cornea or 40 micrograms per square centimeter. Like other sulfhydryl substances glutathione has a high competition factor for mustard and yields a nontoxic compound as the resultant of this reaction. It was of interest, therefore, to discover how much of the mustard absorbed by the cornea reacted with glutathione.

Procedure and Results: Glutathione was extracted from whole beef corneas or from corneal epithelial cell preparations with sulfosalicylic acid and titrated colorimetrically with the aid of the nitroprusside reaction. Previous studies reported elsewhere (3) have shown that this method is adequately specific when applied to this tissue, and that practically all of the measurable glutathione in this tissue is contained in the epithelium. Sulfosalicylic acid was added in such amounts as to constitute 2.3% of the extracting solution. Mustard alone gave no color reaction in the nitroprusside test.

Studies reported in the previous section showed that under the conditions of exposure to mustard which we used, approximately 70 micrograms of mustard were taken up per beef cornea in half an hour.

1) Intact beef corneas were exposed to mustard vapor for ½ hour in the deep cylindrical vessels previously described. Pairs of eyes carefully matched as to corneal size were used for treatment and

controls. After exposure, the treated and control corneas were incubated for one hour and then extracted with sulfosalicylic acid. No significant difference in glutathione content was noted between the treated and control samples, though both showed less glutathione than fresh unincubated specimens.

2) Beef eyes of uniform size were selected for each experiment, and the epithelial scrapings of 4–5 corneas pooled for each sample. This amount of tissue contained about 0.6 mg. glutathione. The scrapings were ground in a mortar and suspended in 4.9 ml. Ringer's phosphate solution, and 0.1 ml. of a solution of mustard in alcohol containing 2

TABLE II

Recovery of Glutathione from Suspensions of Corneal Epithelium in Ringer's Phosphate Exposed to 2 Mg. Mustard for 30 Minutes

CONTROLS GLUTATHIONE ALONE	TISSUE SUSPENSION	TISSUE SUSPENSION + 2 MG. MUSTARD	TISSUE SUSPENSION + GLUTATHIONE	TISSUE SUSPENSION + GLUTATHIONE + 2 MG. MUSTARD
	Milligrams Glutathione Recovered			
Complete recovery	0.48	0.40	3.7	2.0
	0.48	0.43	3.0	1.75
	difference not significant		3.0	1.6
			3.0	1.85
			3.0	1.7
			Av. difference = 1.36 mg. or 45.3%	

mg. mustard was added. In some cases 2 mg. glutathione was also added. Samples and controls without added mustard were allowed to stand for 30 minutes at room temperature, when precipitated with sulfosalicylic acid. A slight, but not significant, disappearance of glutathione was noted in the epithelial cell emulsions treated with mustard, though when glutathione had been added there was marked diminution on exposure to mustard.

3) Similar samples of corneal epithelium were next ground in a tissue grinder with broken glass, suspended in Ringer's phosphate solution, and then treated with 2 mg. mustard. The results in these experiments showed a significant drop in titratable glutathione. Some of these samples were frozen immediately after scraping, thawed

in about an hour, refrozen and thawed again, and then ground with powdered glass in a tissue grinder. Smears from these preparations showed complete disintegration of all cellular and nuclear structure. After exposure to mustard a significant drop in glutathione content was found.

TABLE III

Recovery of Glutathione from Preparations of Completely Mascerated Corneal Epithelium Exposed to Mustard

Corneal Epithelium ground in tissue grinder with glass after freezing and thawing.

TISSUE SUSPENSION ALONE	TISSUE SUSPENSION + 2 MG. MUSTARD	DIFFERENCES
.35 mg.	.20 mg.	.15 mg.
.48 mg.	.30 mg.	.18 mg.
.65 mg.	.40 mg.	.25 mg.
.55 mg.	.41 mg.	.14 mg.
.55 mg.	.20 mg.	.35 mg.
	.20 mg.	

Average difference = .21 mg. or 41%

TABLE IV

Recovery of Glutathione after Treatment with Mustard for 30 Minutes

2 MG. GLUTATHIONE IN RINGER'S PHOSPHATE	2 MG. GLUTATHIONE IN RINGER'S PHOSPHATE + 2 MG. OF AN ALCOHOLIC SOLUTION OF MUSTARD	DIFFERENCE
mg.	mg.	mg.
2	1.10	0.9
2	1.60	0.4
2	1.10	0.9
2	0.95	1.05
2	1.10	1.0
	0.9	1.1

Av. difference = .89 mg. or 44.5%

4) Control experiments were performed with glutathione in Ringer's phosphate solution with and without added mustard. After standing at room temperature for ½ hour sulfosalicylic acid was added and titrations for glutathione performed. The proportion of glutathione which disappeared in these solutions was of the same order as in those of paragraph three above.

It is concluded that in the intact cell glutathione is relatively in-

accessible to reaction with mustard. This might be due to the presence of strongly competing factors in the tissue, but this is unlikely because mere grinding of the cells removes this protection. A second possible explanation is that the glutathione is protected by having its sulfhydryl group blocked through some reversible chemical association in the tissue. This is also unlikely since grinding of the tissue would not be expected to reverse such an association. A third possibility, and one which appears most likely, is that the glutathione normally resides in a region within the cells to which mustard has poor access. This conclusion is the more remarkable when considered in relation to the fact to be shown in subsequent papers of this series, that with the doses of mustard used in these experiments many cellular processes are inhibited 100%. One must conclude that cellular components concerned in these inhibited processes either compete for mustard much more intensely than does glutathione or that they lie in regions much more accessible to mustard. The latter would appear the more likely.

III. ELECTRICAL RESISTANCE OF THE CORNEA

The experiments reported in the previous section strongly suggest that mustard does not distribute itself homogeneously throughout the affected cells but may, on the contrary, present itself in much higher concentrations in some intracellular regions than in others. Since mustard is much more lipoid soluble than water soluble it may be supposed that the initial distribution of the agent would occur through solution in the lipoid components of the cells. Moreover, mustard does not become reactive until activated by water. Consequently, those cellular components adjacent to lipoidal structures might be expected to receive the heaviest dose of the agent. These considerations are supported by the fact that the rate of disappearance of free mustard in tissue is considerably slower than in homogeneous aqueous solutions and is similarly retarded in aqueous emulsions of lipoids such as lecithin.

In view of these possibilities it appeared desirable to determine whether the primary reaction of mustard with the cornea was associated with any demonstrable change in the cell membrane. The electrical resistance of the cornea depends on the impermeability of the

epithelial boundaries to electrolytes and consequently may be used as an index of the integrity of the cell membrane. It is to be admitted that the argument just outlined loses some of its cogency when one considers the fact that the nitrogen mustards which even at physiological pH are much more water soluble than mustard nevertheless produce an almost identical pathological effect. The following experiments were performed in cooperation with Dr. Curt Richter and Mrs. Betty Woodruff.

Methods: Current was applied through silver-silver chloride electrodes connected by a tube of physiologic saline to agar plugs made up in physiologic saline. The ends of the agar plugs which were brought in contact with the tissue were trimmed down to a determined area and shape. The resistance of the electrode circuit and ammeter was less than 200 ohms. Increasing voltage was applied until the current reached 2 milliamperes and the voltage necessary to produce this current was measured by a volt meter and used to calculate the resistance (4).

In preliminary experiments an electrode with a circular surface 2 mm. in diameter was placed in contact with the cornea of each eye of a rat either under ether anaesthesia or freshly killed by decapitation. The resistance measured was about 100,000 ohms. When the epithelium was scraped off one cornea and the electrodes reapplied the resistance dropped to about 50,000 ohms, and when the epithelium had been scraped off both the corneas the resistance was less than 500 ohms. Similarly, when a freshly enucleated eye was placed with one electrode in contact with the corneal epithelium, and one in contact with the sclera, resistance of 50,000 to 200,000 ohms was measured, but if the epithelium was scraped off the resistance was less than 500 ohms. Similar results were obtained when the isolated cornea was placed with its endothelial surface in contact with one electrode, its epithelial surface in contact with the other. It seems clear that only the corneal epithelium contributes an appreciable resistance to the flow of current. No difference could be measured depending on the direction in which the current was applied.

Very large variations in resistance were found to occur with variations in the state of hydration of the tissues. If freshly enucleated rats' eyes were placed in 0.9% sodium chloride solution for 15 minutes

or more the electrical resistance dropped markedly. If the eyes were allowed to dry at room temperature the resistance rose markedly. This was not due simply to the presence or absence of a surface film since a partially dried cornea maintained its high resistance even if the surface was washed with saline immediately before the measurement. In the experiments reported below this variation was largely eliminated by placing the treated and control eyes together in closed moist chambers with the eyes resting on filter paper soaked in physiologic saline solution. Under these circumstances the resistance in the control eyes was stable for an hour or two and then slowly fell. When two eyes of the same animal were compared the resistance was found to be similar (within 30%). In different animals the resistance varied from 100,000 to 500,000 ohms per square millimeter.

Rats' eyes were exposed to mustard vapor either in vivo, or supravitally by suspending freshly enucleated eyes in a chamber saturated with mustard vapor. Exposures were varied up to levels sufficient to produce lesions of maximal severity. For comparison other eyes were exposed to iodoacetate, fluoride, cyanide, and ultraviolet light. Resistance was measured before and immediately after exposure to mustard and to these various other agents, and in no case was any consistent change in resistance noted different from that found in control eyes exposed to solutions or chambers free from toxic agents. On incubation in moist chambers the resistance of both control and treated eyes slowly declined for several hours. When the epithelium began to become loosened after several hours of incubation following exposure to mustard, fluoride, iodoacetate, and ultraviolet light (but not after cyanide) the resistance dropped sharply, and when the epithelium sloughed off the resistance dropped to minimal levels.

It is concluded that exposure to mustard does not result in any primary injury to the corneal epithelial cell membrane detectable by measurements of the electrical resistance of the tissue. The conclusion that the cell membrane is not a primary site of mustard injury was supported by the results of experiments on red blood cells performed in collaboration with Dr. Wintrobe. Neither the fragility nor the sedimentation rate of red blood cells was altered on exposure to mustard in doses as high as 0.1 mg. per ml. Furthermore, Adrian (5) has reported that action currents in nerve fibers exposed to mustard remain normal.

IV. HISTOLOGICAL STUDIES

The following study was undertaken in a further effort to discover the intracellular sites of the primary reaction of mustard. Since mustard has been shown to combine with various reactive groups of proteins it seemed possible that the acid-base characteristics of the reacted proteins might be altered and that this alteration might exhibit iself in some change in the acid-base staining characteristic of the tissue. In order to explore this hypothesis it was necessary to develop quantitatively controlled methods of staining and of measuring the amounts of stain bound by the tissue.

Methods: The eyes of young adult rabbits were used in these experiments. One eye was exposed to saturated mustard vapor, the other eye was used as control. Severe contaminations were produced by exposures of from 30 minutes to 1 hour to saturated vapor at 24°. The eyes were removed thirty minutes later. At this time no marked histo-pathological changes were to be found on routine histological study.

In the first experiments fixation was achieved by a modification of the Altmann-Gersh (6) freezing-drying technique. Immediately on enucleation the tissue was frozen by immersion in absolute alcohol which had been cooled by solid carbon dioxide. The container and tissue were then transferred to the freezing compartment of an electric refrigerator and maintained at a temperature of −11° for 3 days. This procedure produced good fixation and dehydration. However, specimens fixed in Zenker's solution gave the same histological results. Consequently Zenker fixation was used in most of the later experiments.

The tissue blocks were embedded in paraffin, and sections 7 microns thick were cut and mounted on glass slides. The paraffin was removed with xylol, and the sections were then passed through a series of alcohol water mixtures and washed in water. The slides were then immersed in dilute solutions of dyes dissolved in M/16 buffer solutions. Acetate buffers were used for the range of pH 2.0 to pH 5.0, phosphate buffers from pH 5.0 to pH 7.0, and borate buffers from pH 7.0 to pH 13.0. Very dilute solutions of dye, of the order of .004 to .008% by weight, were used. The tissue and dye were equilibrated over a 24 hour period at room temperature, and were then mounted in the dye

solution covered by a cover slip. Estimate of the intensity of the staining was made with the aid of a simply constructed colorimeter attached to a microscope.

This apparatus was constructed with standard microscope lamps, microscope, camera lucida, a rack and pinion, lucite wedge containing solutions of dye, and a slit and focusing lens. The optical system is shown in Fig. 4. A square diaphragm was inserted in the ocular focal

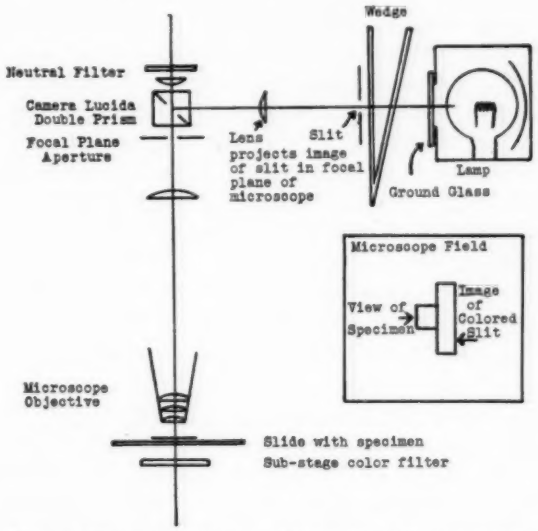

FIG. 4. OPTICAL SYSTEM OF MICROSCOPE-COLORIMETER
Inset shows field as seen through microscope ocular.

plane of the microscope so that only a few cells could be viewed at a time. The camera lucida prism was arranged so that the image of the illuminated rectangular slit could be focused immediately adjacent to the square aperture filled with the images of the cells being studied. The intensity of color in this rectangle could be varied by moving the wedge up and down behind the slit. An arbitrary scale was placed on the rack and pinion so that a numerical value could be given each reading.

Before each set of readings a region of the microscopic slide prepara-

tions which contained no tissue was focused in the field of the microscope and the lucite wedge was raised so that the colorless portion of the wedge below the dye chamber was behind the slit. The microscope lamp, substage aperture, and, if necessary, color filtters were arranged so that a good match in brightness and hue was obtained in the two comparison fields. Some dyes in solution do not give good color matches with the same dyes in histological stains. When this occurred the color in the wedge was adjusted to match that in the sections by using appropriate dye mixtures. For instance, crystal violet in stained sections has a more bluish color than in solutions of the same pH. An appropriate bluish color can be achieved within a small range of variation by lowering the pH or the concentration of the crystal violet solution in the wedge. The same result can be achieved more easily by using mixtures of crystal violet and methylene blue in the wedge. Since we were concerned only with relative color intensities in various sections and various portions of the same sections, no special attention was given to the asolute concentration of the dyes in the comparison wedge, but only a convenient concentration was chosen which would give readings in the middle range of the wedge. The same standard solution, was, of course, used throughout for any given experimental set of readings.

Three readings were taken on each section and two or three sections stained with each dye buffer mixture were read. After all of the slides had been measured the labels were uncovered and the readings for similarly prepared slides were averaged. The sections were stained with either basic or acid dyes, but not with both together. The stains were not "differentiated," i.e., washed in decolorizing solutions, but were mounted without washing in the same dye solution in which they had been stained.

Results: Crystal violet was the basic dye of choice. When normal controls were stained it was found that the intensity of the stain increased in the epithelium and stroma as the pH of the dye solutions was increased (Fig. 5). As the pH increased from 3.5 to 6 metachromasia of the tissue became more marked. When the stainability of control corneas was compared with contaminated corneas no difference could be found at low pH. At pH above 6.0 the staining intensity was so great that accurate readings became difficult. It was, therefore, impossible to extend the study of the basophilic staining of the

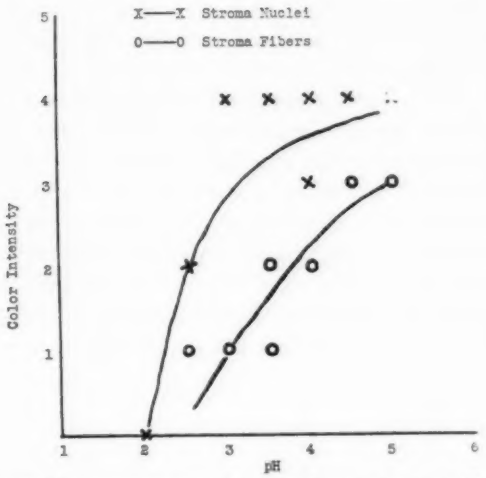

FIG. 5. SAMPLE OF MEASUREMENTS OF STAINING INTENSITY WITH .0047% CRYSTAL VIOLET SOLUTION

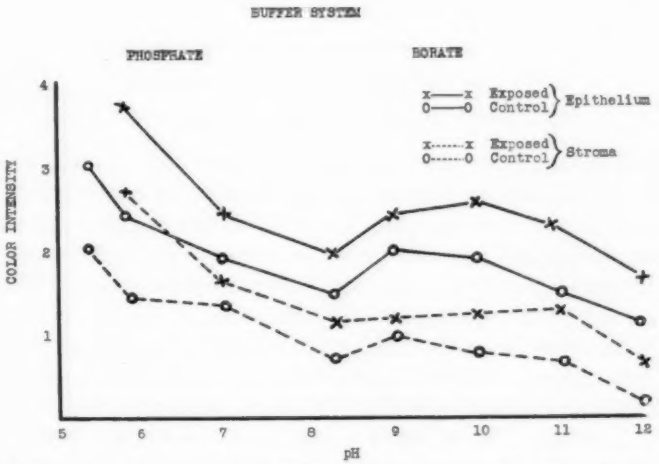

FIG. 6. STAINING INTENSITY WITH .008% PHLOXINE AFTER ONE HOUR EXPOSURE TO SATURATED MUSTARD VAPOR

tissue to the pH regions at which the sulfhydryl groups of proteins might be expected to dissociate.

In order to study the acidophilia of the tissue the eosin family was investigated. Phloxine was selected because the color produced was the most easily readable. Figure 6 shows the intensity of dye in the sections stained at various pH. At pH 5 and below color matching was difficult and the dye in the buffer solutions is partially or completely precipitated. In all sets of experimens with phloxine a broad hump in the curve is observed around pH 10. It would seem likely that in this region the phenolic group of phloxine becomes dissociated and that the resulting dibasic acid has a higher affinity for the tissue than the monobasic acid present at lower pH, though no color change in phloxine solutions is noted in passing through this pH range. Com-

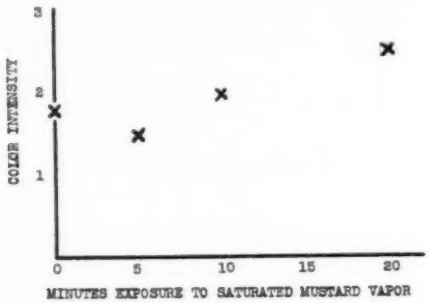

FIG. 7. STAINING INTENSITY WITH .007% EOSIN B AT pH 7.5

paring the graphs of the contaminated issues with those of the controls, it is seen that throughout the pH range studied the contaminated tissue takes up more dye than does the control. The increased stainability is apparent when less severe exposures to mustard are used, but is no longer detectable after only 5 minutes exposure or less (Fig. 7).

In one set of experiments the control and contaminated sections were immersed in a 5% solution of potassium cyanate at pH 7 for 24 hours, rinsed, and then stained with phloxine at pH 6.0 and pH 7.0. The controls showed a smaller dye uptake than controls not treated with cyanate. The mustard contaminated tissues also showed less acidophilia after cyanate treatment than without this treatment, but they still showed the same excess over the controls as in experiments in which cyanate was not used (Table V).

The increased acidophilia of tissues after mustard contamination

indicates that in the reaction of mustard with tissue either new basic groups are formed or acid groups are suppressed. If carboxyl groups in appreciable numbers were suppressed, some decrease in the basophilia of the tissue should have been noted at pH below 6 but this was not the case. Reaction of mustard with sulfhydryl groups would, of course, diminish the number of these groups capable of dissociating to form anionic side chains at pH 8 and above. However, the increased acidophilia is already apparent well below the dissociation point of the common sulfhydryls of the tissues. Furthermore the increased acidophilia is also seen in Zenker fixed tissues. It is most doubtful that any sulfhydryl groups survive in the reduced state after Zenker fixation and the subsequent washing in Lugol's solution which

TABLE V

Intensity of Phloxine Stain in Epithelium Treated with KCNO and Controls

Corneas exposed to saturated mustard vapor for 20 minutes at 22°C. Sections immersed in 5% KCNO at pH 7.0 for 24 hours before staining.

	pH 6.0	pH 7.0
1. Exposed, treated with KCNO	1.4	1.5
2. Control, treated with KCNO	1.1	1.1
3. Exposed, no KCNO	1.6	
4. Control, no KCNO	1.3	

is necessary to remove the precipitated mercury. Consequently, the increased acidophilia of the tissue cannot be attributed to the suppression of SH acid dissociation.

Furthermore, the persistance of the increased acidophilia after cyanate treatment indicates that the basic groups involved are not primary nor secondary amines, and its persistance at high pH indicates that the basic groups involved are relatively strong bases. These considerations lead to the suggestion that the change in the staining reactions of the tissue may be due to the formation of alkyl-sulphonium groups in the tissue in its reaction with mustard.

With the staining technique used control tissues take up considerable amounts of dye, and a difference between mustard treated tissues and contols could be observed only after massive exposures to mustard. With these massive doses no special intracellular locations of enhanced

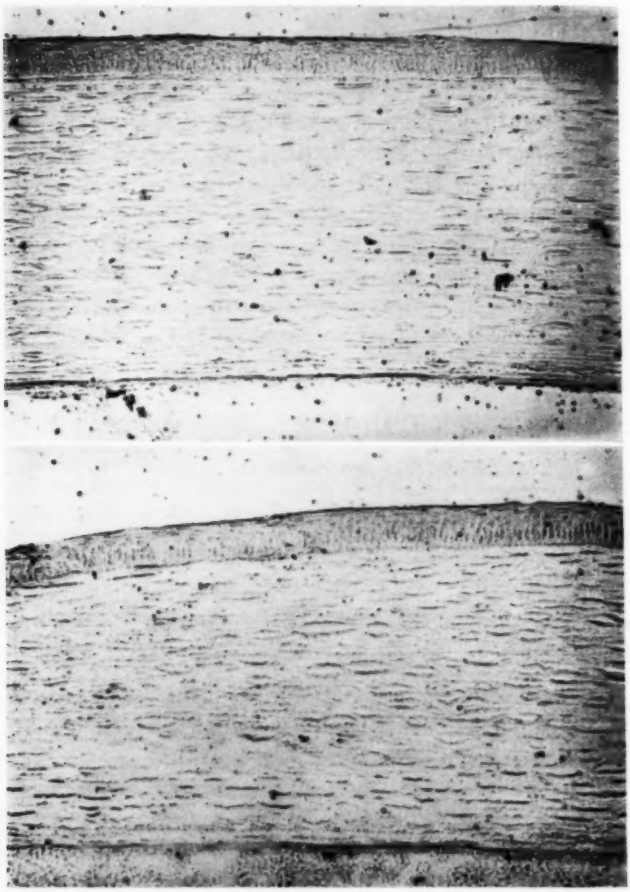

FIG. 8. CORNEAL SECTIONS STAINED WITH PHLOXINE AT pH 10 AFTER PREVIOUS TREATMENT WITH GLYOXAL AND WITH NITROUS ACID

Above: Control.

Below: Exposed to 20 mg. mustard per liter for 20 minutes.

eosinophilia could be recognized. The experiments reported above with potassium cyanate suggested that the non-specific staining of the tissues might be reduced by special treatments leading to an en-

hanced contrast between the mustard treated tissues and the controls. It was found that if the sections were exposed to glyoxal and then to nitrous acid the phloxine binding capacity of the controls was greatly reduced without affecting the excess stain uptake of the mustard exposed tissues. The experiments were performed as follows:—

After removal of the paraffin, sections were placed in a 5% solution of glyoxal in phosphate buffer pH 7 for 24 hours, washed in water and then placed in a solution containing 10 grams KNO_2 plus 10 ml. glacial acetic acid in 80 ml. water, also for 24 hours. After thorough washing they were stained in a .004% solution of phloxine at pH 10, and mounted in this dye solution. Control tissues were almost free of the red color of phloxine but were slightly yellowish presumably as the result of the formation of nitro compounds. When viewed through a yellow color filter they appeared almost colorless. Tissues that had been exposed to saturated mustard vapor for 30 seconds or longer showed an appreciably enhanced acidophilia, but with these doses the stain was still relatively diffuse and no special intracellular sites for the enhanced eosinophilia could be distinguished. The effort was therefore made to push the experiments to the extreme limit of sensitivity of the method.

With the kind cooperation of Dr. Silver at Edgewood Arsenal rabbit eyes were exposed to a concentration of 20 milligrams of mustard per liter for 20 minutes. This is approximately equivalent to an exposure to saturated mustard vapor at 24° for 3 seconds. Exposed and control eyes were fixed in cold alcohol or in Zenkers and sections treated with glyoxal and nitrous acid before staining with phloxine. The difference between the treated and the control specimes was almost imperceptible but the region of the nuclear membrane and the basement membrane appeared slightly more intensely stained in the treated than in the controls, suggesting that these may be among the sites of primary mustard reaction.

Discussion: Estimates have been presented of the amount of mustard taken up by the cornea after various types of exposures. These studies were undertaken as a guide in the interpretation of the experiments that were to follow. In the succeeding papers in this series it will be shown that, as the dosage of mustard is increased, one tissue process after another becomes inhibited, one symptom after another

becomes manifest. More than a thousand fold variation in dosage is required to cover the range from the most sensitive to the most resistant effects. Yet even with massive doses capable of producing complete necrosis of the tissue, some tissue components such as glutathione, which in vitro are among the strongest biochemical competitors for reaction with mustard, still fail to react in measurable quantity.

These facts and the experiments reported above point strongly to the conclusion that mustard may distribute itself within the cells in a highly unequal manner, finding ready access to some regions while other regions are relatively inaccessible to it. Our search for the intracellular sites of most intense mustard reaction has been largely inconclusive. There is no evidence that the cell membrane is particularly susceptible to mustard damage, but there is some evidence, regrettably feeble and inconclusive, that the region of the nuclear and basement membranes may be more intensely attacked by mustard than other portions of the cell.

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III. THE HISTOPATHOLOGY OF THE OCULAR LESIONS PRODUCED BY THE SULFUR AND NITROGEN MUSTARDS*

ALFRED E. MAUMENEE AND ROY O. SCHOLZ

The correlation of histopathological with clinical observation on the ocular lesions produced by the mustard group of agents have been published by a number of authors (1). The present study was undertaken in order to provide an adequate base for the more detailed and specialized studies, reports of which are to follow. It is presented here as a background to those further studies, and because a number of the histopathological observations which this study revealed have not been noted by the earlier investigators cited above.

For the purpose of the present study, doses of the toxic agents were used which cause severe ocular damage. When the lesion was allowed to run its full course following these doses, permanent corneal opacities resulted in almost all eyes, perforation of the cornea in 10–20%. Injuries of this severity by sulfur mustard were produced either by exposing the eye to saturated mustard vapor at 24°C. for 60 seconds or by placing a droplet of 140 micrograms of liquid mustard (Levinstein redistilled 95% pure) on the corneal limbus. Three nitrogen mustards were used for comparison: HN1 or $H_5C_2N(C_2H_4Cl)_2$, HN2 or $H_3CN(C_2H_4Cl)_2$ and HN3 or $N(C_2H_4Cl)_3$. Most of the nitrogen mustard injuries were produced by placing a 3.5 cu. mm. droplet of a 5% aqueous solution of the HCl salt of the agent on the center of the cornea. In one series the eyes were exposed to saturated vapor of the free base of HN2 at 24°C. for 30 seconds. For the vapor exposures the chamber shown in Fig. 1 of the preceding paper (2) was used. The animal's eye was proptosed from its socket before the agent was applied and the lids allowed to close immediately afterwards. The eyes were removed at stated intervals after exposure, fixed in Zenker's solution, sectioned in paraffin and stained with hematoxylin and eosin. Rabbits were used for all of the observations to be reported in this paper.

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

OBSERVATIONS

All four toxic agents produced, with these dosages, lesions of approximately the same severity and similar in their histological characteristics. The lesions produced by all four agents will, therefore, be described together with only an occasional need to refer separately to the different agents.

Conjunctiva: The conjunctival damage resulting from exposure to the mustard agents shows a remarkable degree of uniformity. HN2

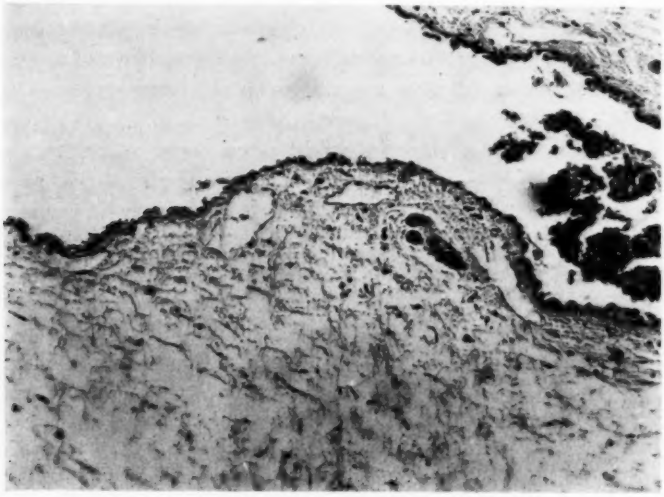


FIG. 1. HN3HCl; 8 hrs. H&E.; 105×. Sloughing of epithelium of conjunctiva.

produces a slightly earlier necrosis of the epithelium and more occlusion of the vessels of the conjunctiva than the other agents. HN1 causes the least severe damage. The number of hemorrhages in the tissue is greatest in HN2. This difference is more obvious on clinical than on histological examination.

Epithelium: The first change noted in the epithelium is a loss of the mucus from the goblet cells. This occurs about 30 minutes after exposure. An exudation of inflammatory cells appears on the conjunctival surface about 24 hours after the injury and continues until the 7th to 10th day. Beginning 8–24 hours after the exposure many

cells in the conjunctival epithelium become pycnotic and loosened from adjacent cells, and slough off (Fig. 1). As this process continues large areas become denuded of epithelium. Regeneration of the epithelium begins on about the 3rd or 4th day and is similar to that seen in the corneal epithelium. At first there is a single layer of irregular large flat cells which migrate into the denuded area, usually arising from crypts or folds that have escaped injury (Fig. 2). During the following week, the epithelium assumes a normal multi-layered

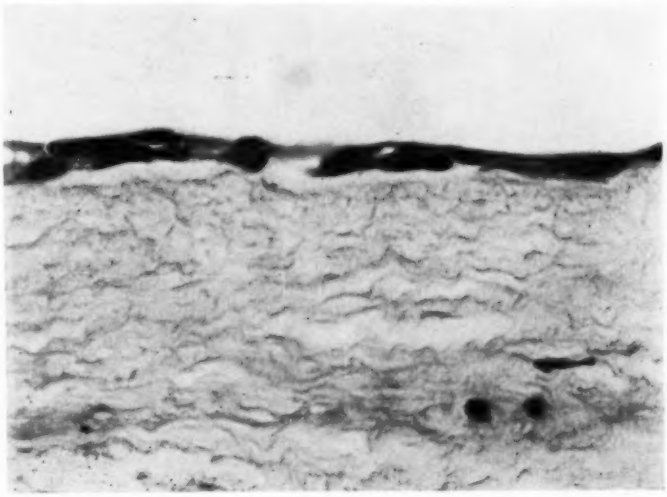


FIG. 2. HN2HCl; 3 days. H&E.; 67×. Regeneration of conjunctival epithelium.

appearance with goblet cells. Even after this occurs, inflammatory cells can be seen passing through the epithelium if the reaction is severe.

Connective Tissue: The first observable effect of the local applications of these agents is a dilatation of the conjunctival vessels. Edema of the conjunctiva is seen in all cases by the end of 15 minutes and becomes steadily more prominent during the first 24 hours. This edema is slightly more noticeable in the early stages after nitrogen mustards than after sulfur mustard. Trypan blue injected intra-

venously during the first half hour after exposure escapes rapidly from the injured conjunctival vessels. During the next two days the interstitial fluid becomes more serous and by the 4th to 5th day it begins to be absorbed. Polymorphonuclear cells begin to infiltrate the connective tissue at 10–18 hours and increase to the 3rd to 7th day, after which they decrease as the general reaction of the eye subsides.

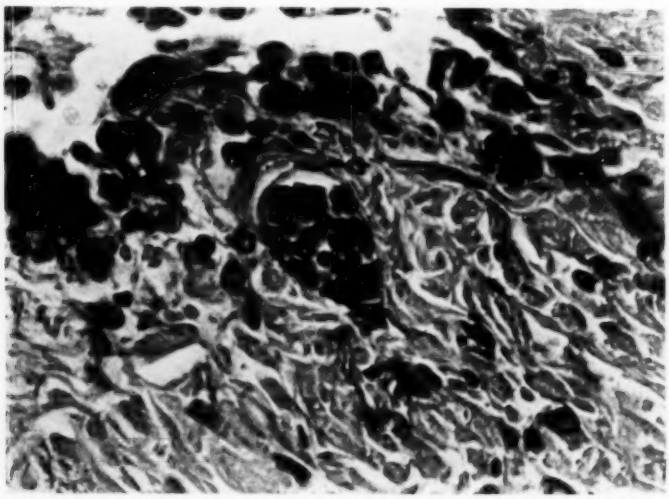


FIG. 3. HN3; 20 hrs. H&E; 670×. Loss of endothelium in conjunctival blood vessel.

Loss of the endothelium of the smaller vessels (Fig. 3) with subsequent occlusion begins at about 20–24 hours. The occluded vessels show densely packed red blood cells but no fibrin clot. At about the same time numerous small hemorrhages occur in the connective tissue. The larger blood vessels show less damage and remain unoccluded in most cases.

In spite of the early necrosis of the epithelium, occlusion of the blood vessels, edema, hemorrhages, and polymorphonuclear infiltation of the conjunctiva there is relatively little residual damage. Occasionally the conjunctival connective tissue becomes thickened and

more fibrous than normal as the acute reaction subsides. Formation of granulation tissue is extremely rare and extensive symblepharon is never found. Occasionally areas devoid of vessels or cells, corresponding to the clinically observed "pearly white" lesions in the conjunctiva, are seen. These lesions are seen as early as the first week after injury and such regions may remain devoid of cells for as long as two years. In the later specimens there is an overgrowth of capillaries in the tissue adjacent to these acellular areas. Larger vessels grow across

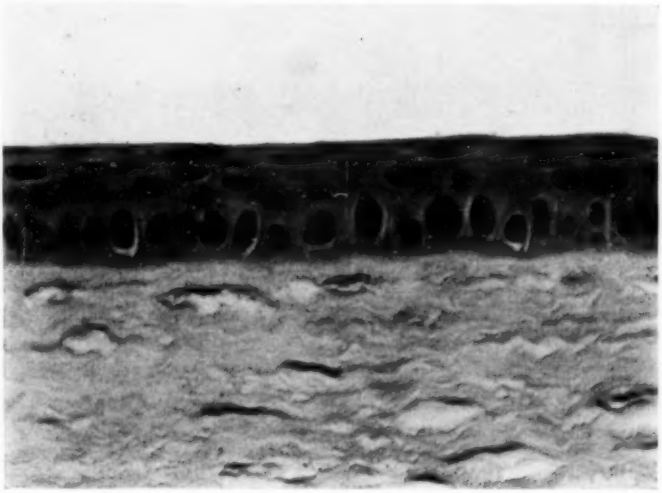


FIG. 4. H&E; 670×. Normal corneal epithelium.

the damaged tissue causing the "marbled" appearance of the conjunctiva.

There is usually slight damage to the nictitating membrane except for necrosis of the epithelium on the surface next to the globe. The large glands on the external surface occasionally lose their mucus and sometimes show squamous metaplasia. The cartilage of the nictitating membrane shows very little change.

Cornea: Epithelium: The sequence of pathological changes in the corneal epithelium following exposure to the mustard agents differs somewhat from that in the conjunctiva. Furthermore, the reaction

is slower in the corneal epithelium and because of this the various steps in the process can be followed in greater detail. The epithelial layer becomes loosened from the stroma a few hours after the injury at a time when many of the cells do not appear histologically to be dead. The time of appearance of the first histological evidence of injury to the cells and the subsequent changes that occur are about the same for all four agents studied.

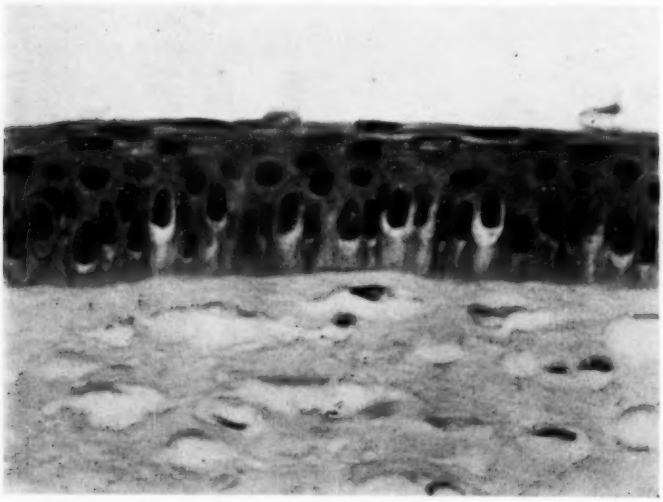


FIG. 5. HN3; 1 hr. H&E; 670×. Migration of basal nuclei of corneal epithelium with cystic changes in basal cells.

The first evidence of damage appears about 10 minutes after a burn. The nuclei of the basal columnar cells move from their normal position at the base of the cell to the more central portion of the cell. At the same time vacuoles appear in the cytoplasm near the nuclei in some cells, while in others the basal portion of the cytoplasm stains less intensely than normally, suggesting an imbibition of water (Fig. 5). The nuclei of the superficial cells, which are normally flat and take a dense homogeneous stain, become swollen so that they are oval, more lightly stained and appear granular. Shortly after this the nuclei of the basal cells begin to swell so that they no longer lie in a straight line

but overlap one another in the sections (Fig. 6). In some specimens about 2 hours after a mild injury scattered nuclei in the basal layers of the epithelium begin to show changes which have been described as fragmentation, and which are discussed in greater detail in one of the succeeding papers (3). The nuclear chromatin first forms intensely stained irregular masses within the nucleus. The nuclear membrane then disappears and the chromatin fragments are dispersed throughout

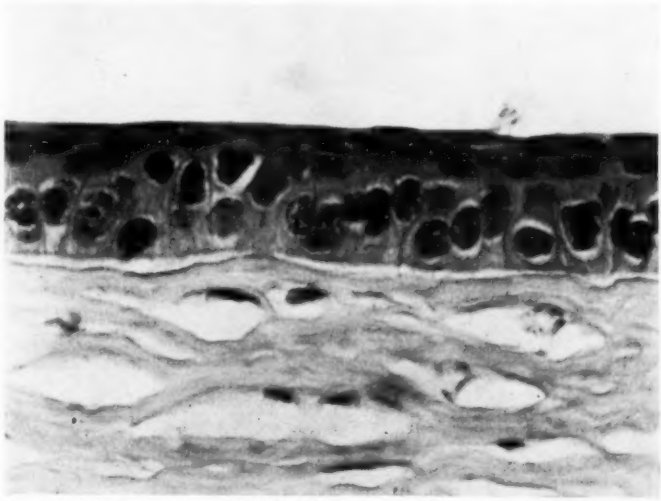


FIG. 6. HN3HCl; 12 hrs. H&E; 670×. Swelling of nuclei in corneal epithelium.

the cell (Fig. 7). Twenty-four hours after a more severe injury a larger number of pycnotic cells can be seen scattered through the epithelium. This is true for injuries by vapor and by the aqueous solutions of nitrogen mustard HCl salts. A slight difference has been noted, however, in eyes injured by a droplet of liquid mustard. In these eyes, a localized area of uniformly pycnotic cells can be seen where the droplet rested (Fig. 8). The adjacent epithelial cells show the changes that have been described above.

After the first 4 hours, the epithelium begins to become detached from the stroma. The line of separation between the epithelial cells

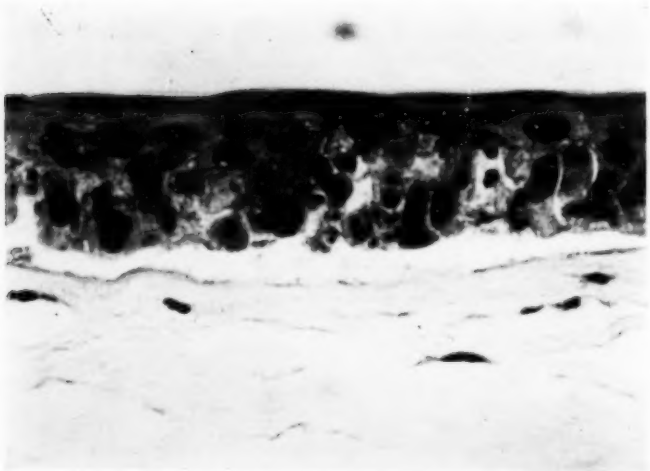


FIG. 7. Mustard vapor; 4 hrs. H&E; 670×. Epithelial nuclear fragmentation.

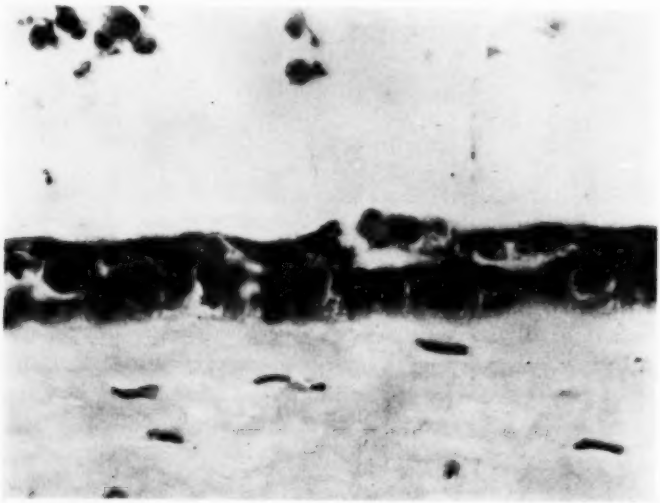


FIG. 8. Mustard Splash; 12 hrs. H&E; 670×. Pycnotic cells in area of splash exposure.

and the stroma occurs either between the epithelial cell membrane and the stroma or just inside of the cell membrane, leaving the basal part of the cell attached to the stroma (Fig. 9). The detachments vary from eye to eye but in general become more marked until 24 to 36 hours at which time most of the epithelium has sloughed off. The phenomenon of epithelial loosening is discussed in greater detail in some of the succeeding papers (4).

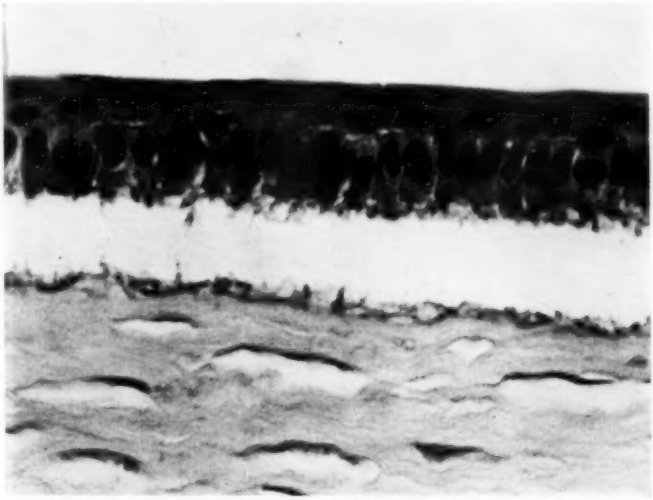


FIG. 9. HN3; 8 hrs. H&E; 670×. Separation of epithelium from stroma.

The time of regeneration of the corneal epithelium varies according to the severity of the injury. The cells are probably derived from two sources: (1) migration of the remaining corneal epithelial cells, and (2) a sliding of the limbal conjunctival epithelium over the cornea. The latter type of repair was first described by Mann and Pullinger (1g) and is frequently seen in mild injuries. Between 24–48 hours the pigmented cells of the limbus begin to move en mass across the cornea forming a grossly visible line (Fig. 10). Frequently epithelium of conjunctival type with goblet cells can be seen on histological examination between this line and the limbus for a few days. These

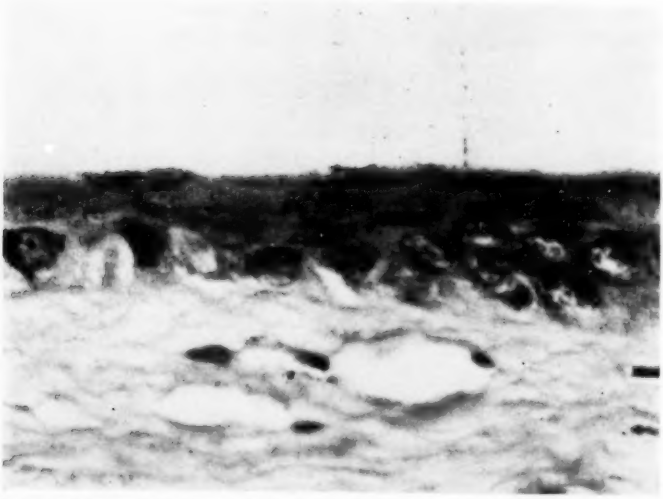


FIG. 10. Mustard Splash; 4 days. H&E; 120×. Migration of pigment line. 4 mm. from limbus.

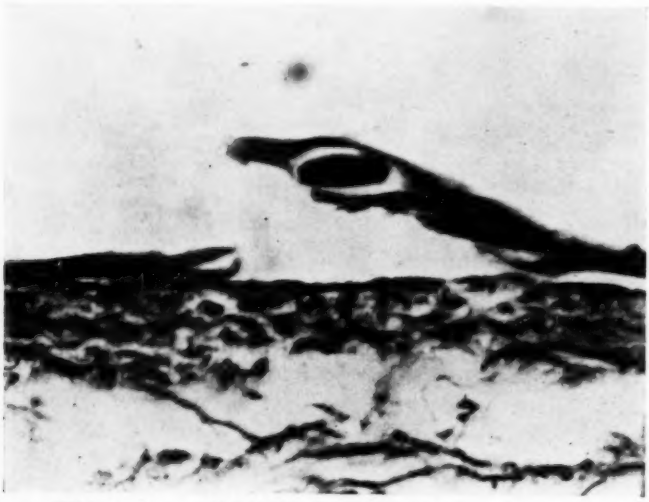


FIG. 11. HN3; 4 days. H&E; 670×. Regeneration of corneal epithelium.

cells later may be transformed into normal corneal epithelium or displaced by regenerating corneal epithelium, and the pigment line disappears from the cornea in a few weeks. In the more severe injuries epithelial regeneration begins on the third or fourth day by a migration of a single or double layer of cells. These cells are large, flat, take a pale stain, and have gaps in their cytoplasm and between the cells. The gaps are probably artifacts due to fixation, but they show that the cells do not have their normal consistency (Fig. 11)

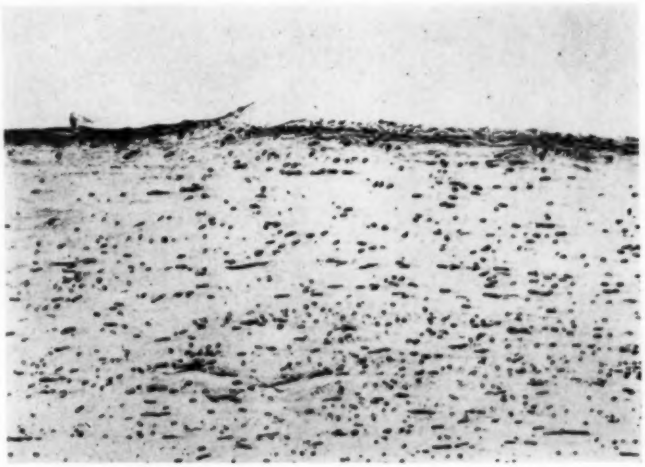


FIG. 12. Mustard Splash; 6 mos. H&E; 150×. Loss of epithelium over heavily scarred and infiltrated corneal stroma.

This thin irregular layer of epithelium usually develops into apparently normal corneal epithelium in a few days if the underlying stroma is normal. However, if the superficial stroma remains edematous or infiltrated with polymorphonuclear cells, the epithelium continues as a thin irregular layer as in the first steps of regeneration and is easily detached from the stroma. This incomplete regeneration resulting, perhaps, from recurrent erosion, has been noted as long as 6 months after a mustard injury (Fig. 12). Frequently in eyes removed 2–6 months after exposure to mustard, the epithelium over heavily vas-

cularized cornea is irregularly thickened with 7 to 8 layers of cells and peg-like processes extending into the stroma (Fig. 13). Occasionally the conjunctival epithelial cells which have migrated over the vascularized stroma fail to be transformed into corneal type of epithelium and retain their normal number of goblet cells. At the point where the vascularization of the cornea stops there is then an abrupt change from the conjunctival type of epithelium to the corneal type (see Fig. 4 of succeeding paper).

FIG. 13. HN1; 5½ mos. H&E; 670×. Thickened epithelium over scarred corneal stroma.

The diameter of the regenerating cell varies up to 30 micra as compared to 10 micra of the normal basal epithelial cell. Thus one regenerating cell may cover 9 times the area previously covered by one cell. Since the migrating cells are only one to two layers thick as compared to four to five cells in the normal epithelium, a remainder of 5–10% of the cells can cover the denuded area caused by the injury.

Stroma: The appearance of the histological damage that occurs in the stroma is also very similar after exposure to any one of the four agents studied. Corneal edema of the "primary type" described by Mann and Pullinger (1f) appears at the end of the first hour in the

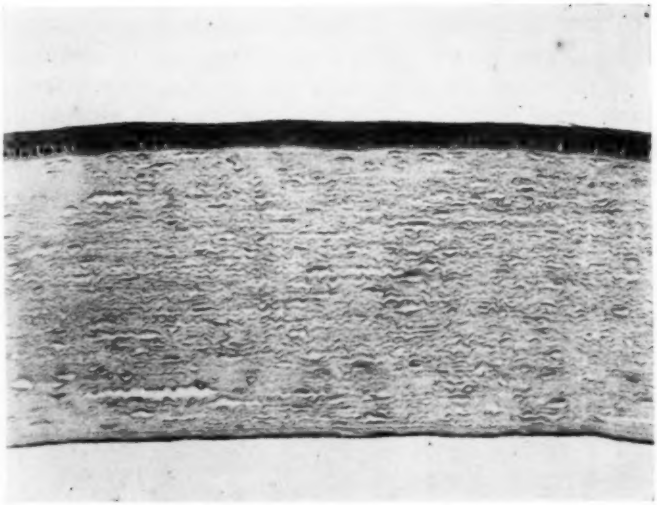


FIG. 14. H&E; 150×. Normal cornea.

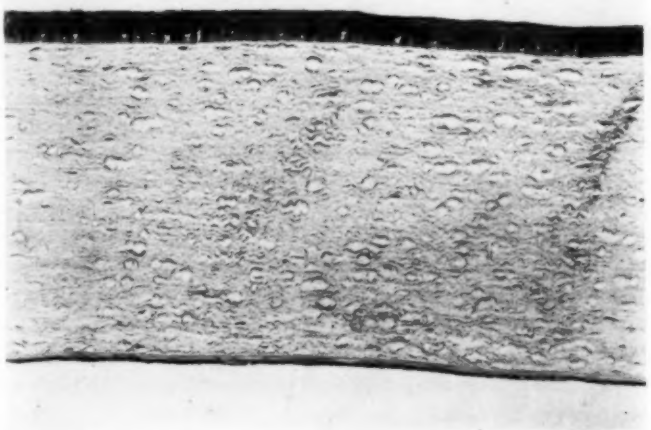


FIG. 15. HN3; 1 hr. H&E; 150×. Edema in posterior part of corneal stroma.

posterior layers of the cornea (Figs. 15, 16). This edema is probably due to an increased permeability of the endothelial cells although they

show no histological evidence of damage at the time. In this respect it is similar to the edema of the conjunctiva which likewise appears before visible histopathological changes in the capillary endothelium. "Primary edema" continues to increase for 24 to 48 hours and the corneal thickness may become two to three times as great as normal. Polymorphonuclear cells begin to invade the anterior two-thirds of the cornea at the end of 24 to 48 hours and continue to increase in numbers until the 3rd to 7th day. The number of invading cells

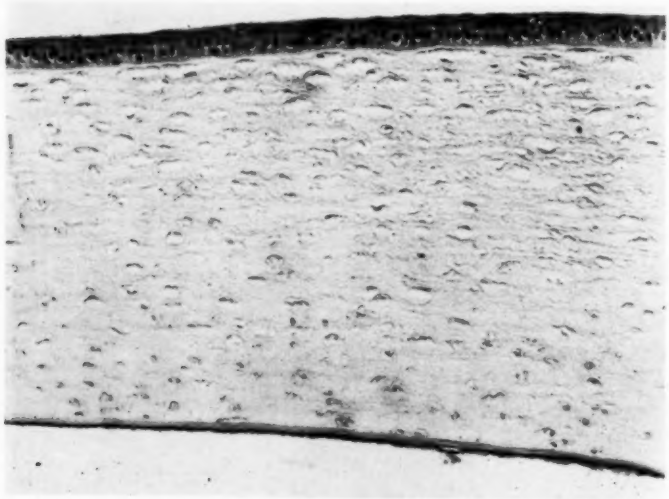


FIG. 16. HN3; 8 hrs. H&E; 150×. Edema of corneal stroma.

depends to a large extent on the number of destroyed corneal corpuscles and also on the intensity of the secondary infection that almost always occurs. As the acute reaction begins to subside on the 7th to 8th day, monocytes can be seen among the inflammatory cells. Pullinger and Mann (5) have suggested that some of the invading macrophages may eventually become fibroblasts and aid in the repair of the stroma. The possibility of such a transformation of macrophages to fibroblasts has been demonstrated by Ebert and Florey (6). At about 4–5 days, blood vessels and fibroblasts also begin to invade the stroma from the limbus and as time advances, areas of typical

granulation tissue may replace the destroyed stroma tissue (Fig. 17). The invasion of the blood vessels and fibroblasts is usually limited to the anterior two-thirds of the stroma. In the posterior part of the stroma many corneal corpuscles usually survive the injury (Figs. 18, 19).

The pathological changes that occur in the stroma cells are more difficult to observe than the changes that occur in the epithelial cells, for even in the normal rabbit's cornea the outline of the stroma cells

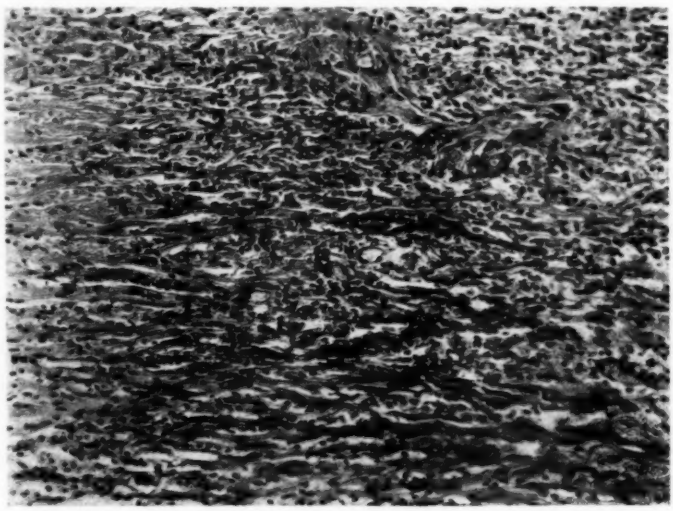


FIG. 17. HN3; 5 days. H&E, 150×. Granulation tissue in corneal stroma.

cannot be seen and the nuclei are thin homogeneous dark blue staining streaks (Fig. 14). Degenerative changes can be seen in the cells of the anterior two-thirds of the cornea, however, as early as 8 hours after a burn. The changes appear either as a clumping of the chromatin material so that the nuclei look like a bit of string with three to four knots tied in it, or the nuclei may swell and disintegrate. In the material studied for this report more cells showed the pycnotic type of degeneration after mustard and more became swollen and disintegrated after HN2. By the end of 24–48 hours most of the cells in the anterior stroma showed one or the other of these changes or had

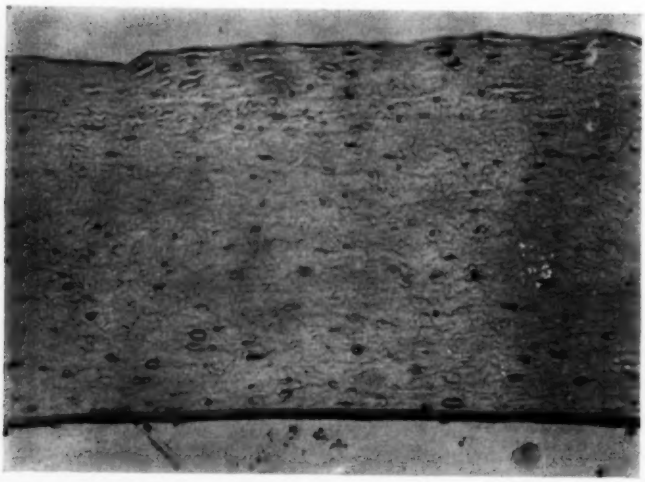


FIG. 18. Mustard Vapor; 24 hrs. H&E; 150×. Pycnosis of cells in anterior stroma and swelling of cells in posterior part of corneal stroma.

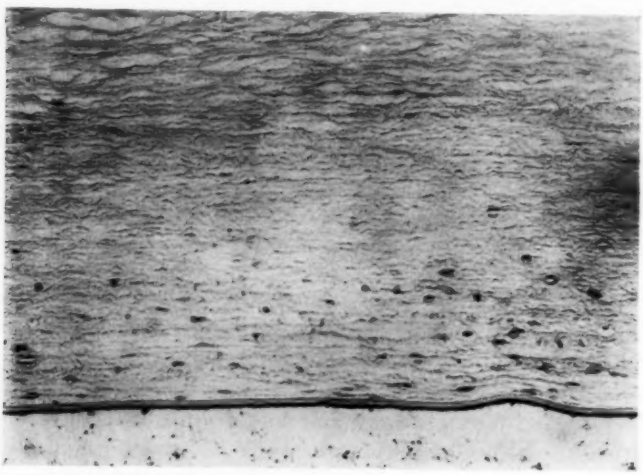


FIG. 19. HN1HCl; 2 days. H&E; 150×. Absence of cells in anterior stroma and swelling of cells in posterior stroma of cornea.

disappeared completely from the tissue (Figs. 18, 19). The cells in the posterior third of the stroma do not appear entirely normal, but

some survive in a high percentage of the eyes studied. If corneal edema is produced by a mechanical injury to Descemet's membrane without applying any vesicant agent, the corneal cells swell, become more clearly outlined and resemble fibroblasts (Fig. 20). During the edema that follows mustard or nitrogen mustard, the corneal cells that remain in the posterior third of the stroma, however, seldom show this picture but have markedly swollen, pale, granular staining nuclei with some margination of the chromatin, indicating that some

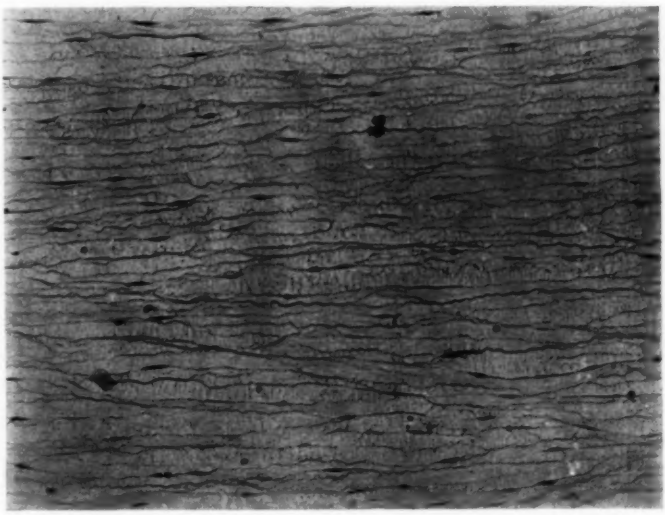


FIG. 20. H&E; 150×. Edema of stroma following mechanical injury to Descemet's membrane.

damage has been done to them other than that produced by edema alone (Fig. 19). These changes have been studied in greater detail and are reported in a succeeding paper (7).

Replacement of damaged stroma cells takes place slowly. The conjunctiva appears almost normal in one to two weeks but vascularization and replacement of the cells in the anterior two-thirds of the cornea is usually just beginning at this time. The inflammatory reaction does not subside nor the cornea return to its normal thickness for two to three months.

In some eyes, blisters of serum containing a few polymorphonuclear and mononuclear cells are found under the epithelium for as long as 3–6 months after injury (Figs. 21, 22). The underlying stroma in these areas is usually heavily vascularized and contains many polymorphonuclear cells. The epithelium is loosely attached and may easily slough to form an ulcer. These areas are probably one of the causes of recurrent ulcerations of the cornea following mustard injury. Long after primary healing, in some eyes in which the cornea has regained complete or almost complete normal clarity areas may be

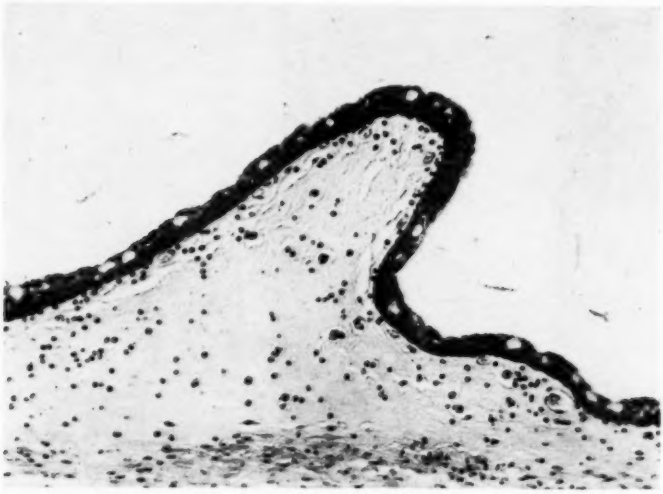


FIG. 21. HN3HCl; 4 mos. H&E; 150×. Edematous bleb under corneal epithelium.

found in the stroma that are still completely devoid of cells (Fig. 23). These acellular areas may be as much as 2–3 mm. in diameter and $\frac{1}{3}$ to $\frac{1}{2}$ the thickness of the cornea. It is possible that these lesions are analogous to the "pearly white" lesions in the conjunctiva in that the fixed tissue cells are destroyed and are not replaced either by regeneration or by scar formation. It seems likely that these acellular areas may also account for some of the late recurrent ulcers. Accumulations of cholesterol crystals as described by Mann and Pullinger (1f) have only occasionally been seen in our specimens and

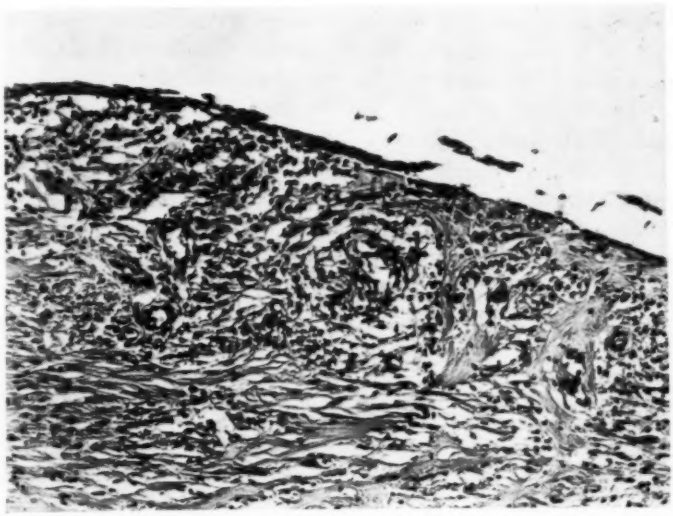


FIG. 22. HN1; 3½ mos. H&E; 150×. Inflammatory reaction in scarred corneal stroma with loss of epithelium.

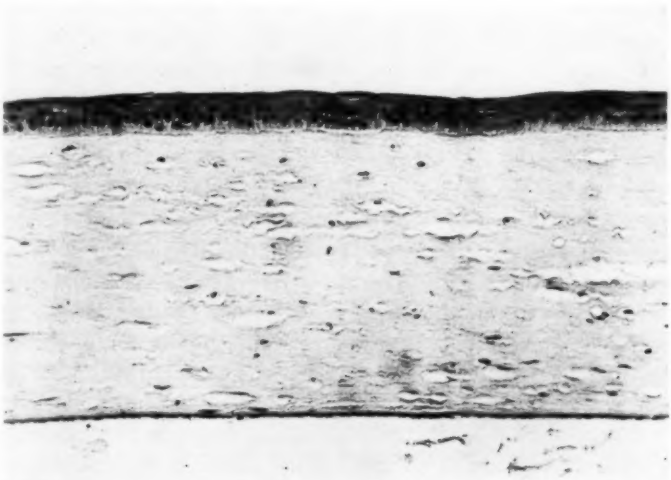


FIG. 23. HN2 vapor; Treated with sodium diethyldithiocarbamate; 21 days. 150×. Clinically clear cornea. Marked reduction in number of stromal cells.

the epithelium over these areas appeared normal. Mann and Pullinger attribute late ulcerations to the presence of these crystals but we have been unable to correlate recurrent erosions with the presence of these crystals.

During the first few days after the application of any one of these toxic agents, the clinical symptoms are dominated by the reactions which occur in the conjunctiva. During this period the severity of the reaction to a particular dose of the agent is remarkably uniform and reproducible from rabbit to rabbit. The corneal symptoms that occur during this period, loss of the epithelial and endothelial coverings, primary edema, and beginning polymorphonuclear infiltration are also relatively uniform. After the 3–4 day, however, as the conjunctival lesions begin to recede, increasing variability in the course of the reaction appears. The same dose that leads to perforation of the cornea in some eyes may result in little or no final corneal opacity in others. This variability in the ocular reaction to mustard is much greater than that found after exposure to some other toxic agents, for instance lewisite. It seems clear that most of this variation concerns events which take place in the corneal stroma.

Part of this variation can be attributed to differences in the intensity of secondary infection, but this by no means accounts for the extent of the scatter. Maumenee and Guyton (8) studied a large series of rats all exposed to as nearly as possible the same dose of mustard and treated with antibiotics. In spite of the fact that the conjunctival bacterial flora was kept at minimal levels throughout the experiment in these eyes, the severity of the reaction varied almost as widely in these animals as in those not treated with antibiotics.

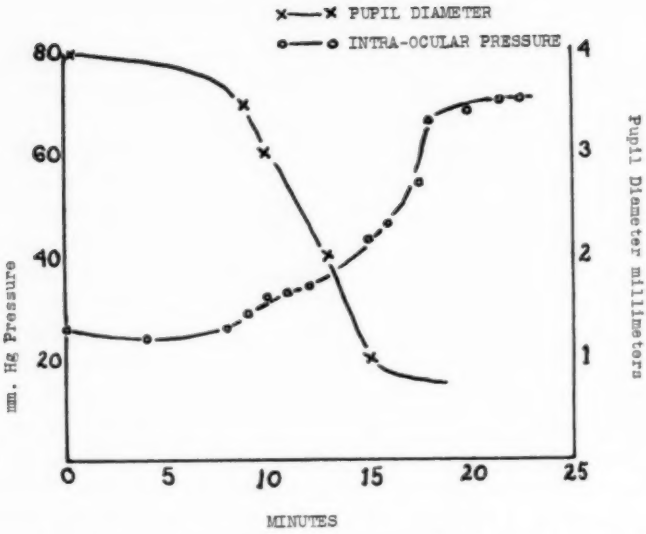
One factor which appears to be correlated with this variation is the variation in the number of dead corneal stroma cells. The intensity of polymorphonuclear infiltration in the cornea, which reaches a maximum about one week after the injury, is closely connected with the number of dead cells. There is, moreover, a close correlation between the intensity of polymorphonuclear infiltration and the liquefaction and disorganization of the corneal lamallae which develops during the second and third weeks. Finally, residual scarring of the cornea is greatly increased if the lamallae have become disorganized so that invading fibroblasts and regenerating keratoblasts are not laid down

in parallel rows. Wide variation in the number of lost or fatally injured cells in the corneal stroma can be recognized in sections as early as the second or third day after exposure.

Endothelium: In contrast to the wide variation in injury to the corneal stroma cells, for a given dose of a given one of these toxic agents, the reactions in the endothelium are much more uniform, but there is a wide variation in relation to variations in dosage and in respect to the four different toxic agents that are being compared. With doses that produce approximately equal reactions in the conjunctiva and corneal stroma, the effect on the endothelium is most to least marked with the agents in the order HN2 > HN3 = Mustard > HN1. After small doses the endothelium shows very little histological change, but some damage must occur as evidenced by the increased permeability of the cells resulting in corneal edema. In all injuries with the standard doses the edema of the posterior cornea develops within 8–10 hours after the injury, before there is a sloughing of the endothelium. With HN2 the edema begins as early as one hour after exposure. Sloughing of the endothelium after exposure to HN2 is first seen about 8–24 hours after exposure. Similar changes appear as late as 3 to 4 days after HN1. The other two agents fall between these extremes. Fragmentation of the nuclei of the endothelial cells has been observed in supravital preparations (3) but was not noted in this study. Regeneration of the endothelium is markedly dependent on the depth and severity of the injury. It is very much slower than the regeneration of the epithelium and begins about 10–14 days after the injury. A marked inflammatory reaction in the anterior chamber with wandering cell deposits on Descemet's membrane seems to retard this regeneration.

Aqueous: An increase in the protein content of the aqueous occurs early in all cases but is more marked with HN2. The cellular reactions depend on the dosage of the agent and extent of damage to the cornea. Strangely, there are only slightly more cells in the aqueous after HN2 than after the other agents.

Iris and Ciliary Body: In the iris again HN2 produces by far the greatest damage. The pupil constricts rapidly following application of this agent. Coincidental with this constriction the intraocular pressure rises (Graph 1). The earliest damage visible histologically



GRAPH 1. PUPILLARY DIAMETER AND INTRAOCULAR PRESSURE FOLLOWING LOCAL APPLICATION OF HN2

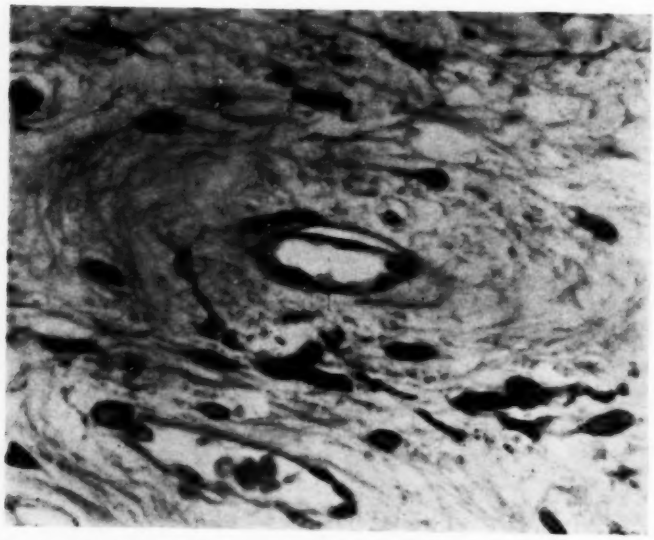


FIG. 24. HN2HCl; 3 hrs. H&E; 670×. Detachment of capillary endothelium in iris.

FIG. 25. HN2 Vapor; 4 days. H&E; 150×. Hemorrhages in necrotic iris.

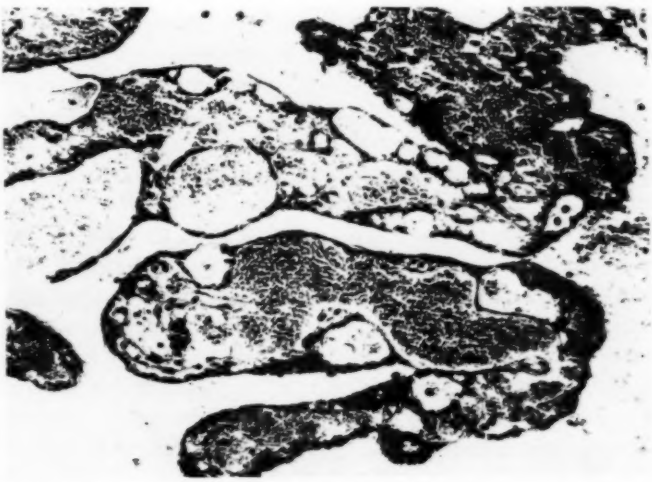


FIG. 26. HN2 Vapor; 4 days. H&E; 150×. Hemorrhages and Greef vesicles in ciliary processes.

in the iris is a detachment of the capillary endothelium (Fig. 24). The nuclei of the detached endothelium do not stain as intensely as

normal. Later the endothelium disappears and the capillaries become occluded by packed red blood cells without fibrin clots. Occlusion of the capillaries and hemorrhages occur as early as 8 hours. On the third or fourth day the iris usually shows extensive necrosis (Fig. 25). The other agents produce occasional small iris hemorrhages and desquamation of capillary endothelium. Mustard and HN1 show the least damage to the iris. All four agents produce Greef vesicles on the ciliary processes within one half to one hour after exposure. HN2 again produces the most marked changes in the ciliary processes (Fig. 26). Greef vesicles appear with 5 minutes after application of this agent.

Lens: Clinically a diffuse clouding of the lens can be observed several weeks after contamination with HN2. The lens was not examined histologically in this study.

DISCUSSION

Injury: The conjunctiva shows marked changes in the early stages of injury in the form of edema of the stroma, loss of the endothelium and occlusion of the small capillaries and necrosis of the epithelium. In the corneal epithelium the first changes to be noted are a fragmentation of some nuclei in the basal layer of cells and then a detachment of the epithelial cells from the underlying stroma. Later some nuclei became pycnotic and the epithelium sloughs off. The first manifestation of damage to the stroma is an edema of the posterior portion which occurs as early as one hour after injury in the HN2. This is probably due to an increase in the permeability of the endothelium. The nuclei of the stroma cells become pycnotic or swell and burst as early as eight hours after damage. Polymorphonuclear cells begin to invade the stroma at about 24 hours. The extent of this type of infiltration depends on the number of stroma cells which have been killed and on the intensity of the secondary infection present. The first histological evidence of damage to the endothelium of the cornea is a sloughing of the cells. The time of occurrence of these lesions varies considerably with the agent used. It occurs as early as 8 hours after HN2 and as late as 3–4 days after HN1. Damage to the iris is relatively mild after the application of HN1 or mustard. However, after HN2, and occasionally and to a lesser degree after HN3, there

occurs a loss of the endothelium of the capillaries with subsequent occlusion of the vessels and necrosis of the iris. Greef vesicles appear on the ciliary processes after the application of all four agents. In addition to these vesicles, hemorrhages occur in the ciliary processes after HN2.

Repair: The conjunctival epithelium is repaired by migration of the uninjured epithelial cells from adjacent areas. The edema is absorbed, and the necrotic debris is removed by the macrophages. New formed capillaries reestablish the circulation when there has been extensive damage to the blood vessels. Occasionally necrotic areas remain acellular over long periods of time in the regions where "pearly white" areas are observed clinically. Markedly dilated vessels can also be observed in some eyes. Extensive areas of granulative tissue and symblepharon are almost never found.

The areas denuded of corneal epithelium are covered by a sliding of adjacent uninjured corneal epithelial cells and by a migration of the conjunctival epithelial cells. The migration of the pigmented epithelium of the limbus across the cornea, and the presence of conjunctival goblet cells over the corneal stroma, is proof of the latter type of repair. If the stroma underlying the new epithelium is normal, the new cells will transform themselves into typical corneal epithelial tissue, or are replaced by corneal epithelium. If, however, the stroma under these new cells is edematous or is invaded by blood vessels, then the epithelium will either remain as a thin sheet sometimes preserving evidences of its conjunctival origin, or become thicker than normal, and may slough off frequently.

The repair of the corneal stroma is somewhat more complicated than the corneal epithelium or the conjunctiva. The mode of repair depends on the extent of the damage done. If a relatively mild lesion is produced in the center of the cornea a few polymorphonuclear cells will invade the stroma and remove the dead corneal cells. A few days later large wandering mononuclear cells, probably macrophages, are seen in the area of the lesions. The dead corneal cells are probably replaced by a transformation of these macrophages into keratoblast or by a migration of uninjured stromal cells. As the corneal edema subsides relatively little opacification will remain after this type of lesion. If a more severe injury is produced a greater number of

polymorphonuclear cells will invade the stroma and there will be a destruction of the corneal lamallae. A few days later blood vessels will begin to invade the stroma along with or just after the macrophages. Numerous fibroblasts are seen in the regions of the new vessels. The repair does not take place in an orderly fashion along the old corneal lamellae. Areas of typical granulation tissue are seen in some places and in other places areas of edema persist for a year or more. This type of repair always results in greater scarring than the previously mentioned type of repair. Some areas may remain acellular for a very long time.

Repair of the endothelium is markedly dependent on the depth and severity of the injury produced. It is very much slower than the epithelium and begins 10–14 days after injury. The endothelial cells are replaced by a migration of the uninjured cells.

Repair of the iris is essentially the same as that of the conjunctiva.

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IV. EFFECTS OF MUSTARD AND NITROGEN MUSTARD ON MITOTIC AND WOUND HEALING ACTIVITIES OF THE CORNEAL EPITHELIUM*

JONAS S. FRIEDENWALD, WILHELM BUSCHKE, AND ROY O. SCHOLZ

The data reported in a previous paper serve to indicate the complex intracellular distribution of some of the bound mustard. Between the initial reaction of mustard with the tissue, and the consequent death of the cells, there is a gap in our knowledge so great as almost to defy analysis. As a first step in the attack on this problem we have assumed that there are many different physiological mechanisms within the cell that are capable of being damaged by exposure to mustard, and have attempted to isolate for study those physiological activities that are experimentally approachable. If the inhibition or malfunction of some intracellular physiological mechanisms could be connected with cellular death, the gap between the biochemical events of the primary reaction of mustard with tissue and the pathological events of cellular death might be somewhat narrowed.

One normal activity of the cornea is the mitotic division of cells in the corneal epithelium. Recent studies in this laboratory (1) have shown that the rat's corneal epithelium is a favorable test object for the study of mitotic activity, and as a result of these studies convenient methods of assay were readily available. Some suggestion that mitotic activity might be particularly susceptible to inhibition or malfunction after exposure to mustard was implied in the report of Berenblum (2) that mustard acted as an antagonist to carcinogens on the rat's skin.

The techniques used for the assay of mitotic activity in the corneal epithelium have been reported elsewhere (1). The basic data which these studies revealed regarding the mitotic activity of the corneal epithelium of the rat are as follows: The corneal epithelium in animals of 50 to 150 gram weight contains approximately 2,000,000 cells, of which approximately 1,400,000 are in the two basal layers. Between 5,000 and 6,000 cells are normally found in mitosis. The duration of

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

the mitotic cycle of a single cell is about 70 minutes. The average duration of the resting stage between mitoses is 7–10 days. The mitoses occur exclusively in the two basal layers and are slightly more numerous near the periphery of the cornea than at the center. Two drugs which have been found most useful in analyzing the mitotic activity are colchicine and ether. Colchicine causes an arrest of mitosis in metaphase and hence an accumulation of mitoses with the passage of time. In proper dosage it apparently has no effect on the rate at which cells enter mitosis. It may be used, therefore, to determine the number of cells entering mitosis during a given period. Brief ether anesthesia stops the entrance of cells into mitosis for a period of 1 to 2 hours but does not, in proper dosage, influence the progress of cells through the mitotic cycle once mitosis has begun. It may be used, therefore, to determine whether the progress of cells through the mitotic cycle is normal or delayed.

In the present study, attention has been focussed chiefly on the nitrogen mustard $H_3CN(CH_2CH_2Cl)_2$ commonly called HN2, but it may be said in advance that qualitatively similar results have been obtained with mustard and with other nitrogen mustards.

EFFECT OF LOCAL ADMINISTRATION OF HN2 HYDROCHLORIDE

In a preliminary study it was found that the local toxic effects of freshly prepared solutions of HN2 hydrochloride instilled in the eye were indistinguishable from those produced by exposure to the vapor of the free base. In the present study aqueous solutions were used unless otherwise specified. The solutions were either freshly prepared or freshly diluted from a 1% stock solution of HN2 hydrochloride in M/1000 HCl. The stock solution was kept on ice and renewed every two weeks. A single drop (approximately 0.05 ml.) of the solution was instilled into one eye of a 50–150 gram rat and the lids held open for 30 seconds after the instillation. At various intervals after the instillation the animals were sacrificed and the number of mitoses counted in the treated and untreated corneas. Counts were made on

a meridional strip of each cornea constituting $\frac{1}{60}$ of the total area of the cornea. The average normal number of mitoses in such a strip is 100–120. Since there is considerable individual variation in mitosis counts on different rats, but little normal variation between the two eyes of the same rat, the numbers given in Table I are the ratio of mitoses in the treated versus the untreated eye of the same rat. In general, the number given is the average of two or more animals for each observation.

TABLE I

Per Cent of Mitosis in Treated Versus Control Eyes

CONCENTRATION OF HN2 HYDROCHLORID	TIME AFTER ADMINISTRATION			NUCLEAR FRAGMENTS	LOOSENING OF EPITHELIUM
	3 hrs.	6 hrs.	24 hrs.		
.25%	43	2	0	+	+
.125	9	0	0	+	+
.06	8	8	0	+	±
.03	4	0	0	+	0
.015	15	43	0	+	0
.0075	24	20	10	+	0
.0037	32	35	6	+	0
.0175		38	33	0	0
.0008		36	52	0	0
.0004		53	52	0	0
.0002			84	0	0
.00005			134	0	0
.000012			147	0	0
.000003			110	0	0

Thresholds: A single drop of 1% HN2 hydrochloride produces a severe lesion in a rat's eye. 0.5% produces a slight lesion. With 0.25% few symptoms are clinically visible; however, histological study reveals during the first 24 hours areas of loosening of the corneal epithelium and some leucocytic infiltration of the tissue. At a concentration of 0.06%, no changes whatever are noted on routine histological study. 0.1% therefore, represents the threshold of local necrotizing action. In human beings pain, lacrimation, and photophobia, associated with fine punctate opacities in the epithelial layer of the cornea, are seen with exposures insufficient to elicit such severe clinical symptoms as those which were observable in the rat.

The administration of HN2 hydrochloride in concentrations between 0.25% and 0.0004% results in a marked decrease in the number of mitoses in the corneal epithelium. The onset and duration of this period of decreased mitosis varies with the dosage. At still lower concentrations there is a slight increase in the number of mitoses in the treated as compared with the untreated eye. A comparison of the mitosis inhibition produced by various toxic agents is given in Table II. The absolute amounts of these substances administered is of no special significance since the toxic agents were dissolved in

TABLE II

% Mitosis Inhibition 24 Hours after Administration

SUBSTANCE CONC. %	HN1	HN2	HN3	MUSTARD	LEWISITE
0.25		100	100		90*
0.12		100		*	42
0.06	*	100*	100*	98	34
0.03		100			15
0.015		100	100		
0.008		90		99	
0.004	87	94	85	93	
0.002	3	67	73	74	
0.001		48		25	
0.0005		48			

HN1 = $H_3C_2N(C_2H_4Cl)_2HCl$ dissolved in water.
HN2 = $H_3CN(C_2H_4Cl)_2HCl$ dissolved in water.
HN3 = $N(C_2H_4Cl)_3HCl$ dissolved in water.
Mustard = $S(C_2H_4Cl)_2$ dissolved in hexane.
Lewisite = $(ClCH=CH)AsCl_2$ dissolved in triacetin.
* = threshold concentration for clinically visible effects.

different solvents and the amount taken up by the cornea no doubt varies with different agents and different solvents. For comparison between the different agents the relation between the threshold dose for the production of clinically visible symptoms and the threshold dose for inhibition of mitosis seems more significant. These thresholds are indicated in the table. It is evident that mustard and the three nitrogen mustards studied produce essentially similar effects, the threshold doses for mitosis inhibition with these agents being about 1/100 the threshold dose for clinically recognizable symptoms.

Fragmented Nuclei: Returning to the HN2 series which was studied in greater detail, it was found that while normal mitoses are almost completely absent in many of these preparations, a number of them show scattered cells in which the chromatin is fragmented and the cell disintegrating. The appearance of these cells closely resembles similarly disintegrating cells seen after colchicine poisoning. Occasionally a few cells with this type of disintegration are found even in normal controls. They are found almost exclusively in the basal layers of the epithelium. The number of these disintegrating cells is generally relatively small. This phenomenon of nuclear fragmentation and cellular death produced by mustard and nitrogen mustard injury has been studied in some detail. The results of these studies are reported in the next paper.

ONSET OF MITOTIC INHIBITION

The normal time required for the completion of mitosis by the corneal cells is about 70 minutes. If the inhibition of mitosis by HN2 were immediate all normal mitoses would be expected to disappear within this period after the administration of the agent. Allowing a short time for penetration and reaction of the agent, this is approximately the case when concentrations of HN2 hydrochloride are used that are near the necrotizing or vesicant level. With much lower concentrations, near the threshold of mitosis inhibition, the number of mitoses in the cornea is found to decline very much more slowly. Thus for example, with a concentration of .0037%, the number of mitoses reaches a minimum only after 18 hours (Fig. 1).

This slow decline in the number of mitoses might be due either to a slow development of the inhibition following the initial reaction of HN2 with the tissue, or to a slowing down of the progress through the mitotic cycle of those cells that were in mitosis at the time of action of HN2. In order to test this possibility, we have made use of the effect of ether anesthesia previously reported. It had been shown that a brief anesthesia of 10 minutes duration is followed by a period of over an hour during which no new mitoses appear in the cornea. During this period of temporary inhibition, those cells that are in mitosis at the time of anesthesia progress apparently quite normally through their mitotic cycle as is shown by the fact that cells in prophase, in metaphase, etc. disappear in orderly fashion from the picture.

The following experiment was, therefore, performed. One drop of 0.0037% HN2 hydrochloride was instilled into one eye each of a number of rats. As shown in Figure 1, this dose leads to a slow decline in the number of mitoses in the cornea. At various times during this slow decline, the animals were anesthetized for 10 minutes with ether. One hour later they were killed by decapitation and the eyes removed

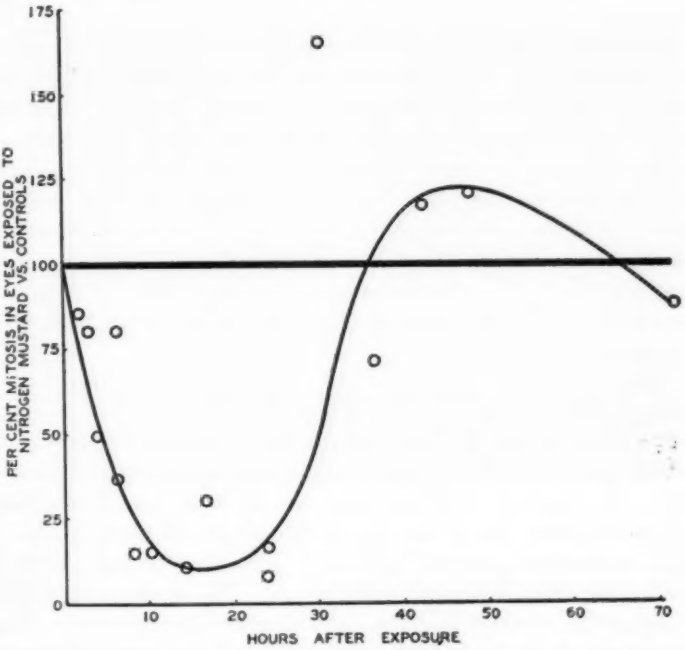

FIG. 1. INHIBITION OF MITOSIS FOLLOWING INSTILLATION OF ONE DROP OF .0037% NITROGEN MUSTARD SOLUTION

for mitosis counts. It was found that the disappearance of mitoses following anesthesia was just as complete in the treated as untreated eye. There is, therefore, no reason to suppose that HN2 in this concentration decreases the rate of progress of cells through mitosis once mitosis has begun. It is concluded, therefore, that the slow decline in the number of corneal mitoses following the administration of very low concentrations of HN2 is due to a slow development of

the inhibition and that this inhibition reached maximum long after the reaction of HN2 with the tissue has run its course.

The implications of this finding in respect to the mechanism of mitosis inhibition require further exploration. One possible explanation may, however, be suggested at this time, namely that HN2 may not directly attack the mitotic mechanism itself but may inhibit the production in the tissue of some substance required for the initiation of mitosis.

With much higher doses of HN2 some slowing down of the mitotic cycle was actually observed. If 0.25% HN2 hydrochloride is instilled into one eye of a rat, the animal then exposed to 10 minutes light ether anesthesia, and the animal sacrificed one hour later, the control eye shows a negligible number of mitoses, while the eye that had been exposed to HN2 still shows an appreciable number. In fact, with this dose of HN2 the last stragglers in the array of mitosing cells have not completed their mitotic cycle even at the end of 3 hours, as is to be seen in Table I. The slowing down of the mitotic cycle with such very large doses of HN2 appeared to affect all phases of the mitosis about equally.

RECOVERY FROM INHIBITION

Spontaneous recovery from the inhibition of mitosis occurs in the corneal epithelium after a lapse of time which varies with the dose of HN2. In Figure 1 it is seen that this recovery can be associated with a transitory rise of the mitosis rate above the normal level. A similar transitory excess of mitoses is seen after recovery from inhibition by ether and some other agents. The larger dose of HN2, the longer the period of inhibition and the slower the recovery. When concentrations as high as 0.03% are used mitotic activity is suppressed for 7–10 days.

CELLULAR GROWTH IN THE ABSENCE OF MITOSES

If a drop of 0.03% HN2 hydrochloride is instilled into rats' eyes every five days, mitoses can be suppressed in the cornea for as long as a month. Throughout the first 2–3 weeks of such an experiment the rats' corneas appear grossly quite normal. By the fourth week, however, some clouding of the cornea and congestion of the conjunctiva

develop along with a moderate mucopurulent discharge. Some of the eyes in this experiment were removed for histological study 10 days after the onset of the experiment. Flat preparations of these corneas, Fig. 2, revealed that the horizontal dimensions of the basal cells and of their nuclei had increased to about double the normal, the area covered by each cell being about four times that of the normal controls; thus the number of basal cells in these eyes was about one

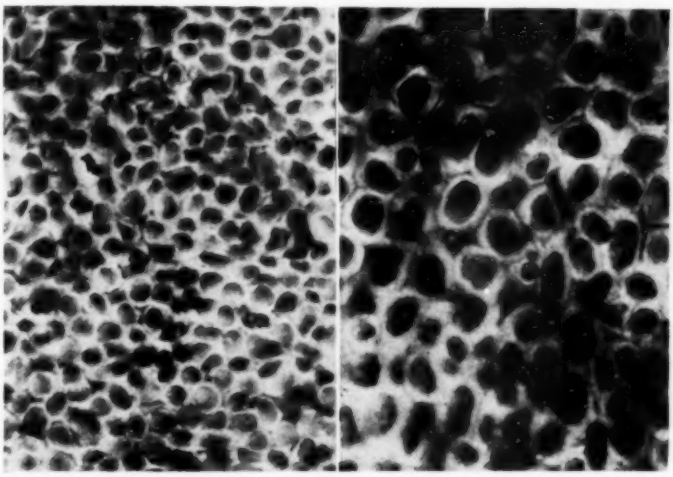

FIG. 2

a) Normal control showing several mitoses.

b) Enlargement of corneal cells after 10 days inhibition of mitosis by nitrogen mustard. This microphotograph was taken under the same magnification as Fig. 2a.

quarter that in the normal, and, since the superficial layers of cells had largely desquamated off, the number of epithelial cells was reduced to less than a quarter of those normally present.

Sagittal sections through some of these specimens revealed that the height of the basal cells was about one half that of the normal. With four time the area and half the height the volume of these cells was about twice that of those in normal controls. Since the average intermitotic period in the basal cells of the rat's corneal epithelium is

normally about 7–10 days, it is evident that the growth of cell volume during this 10 day period of mitotic inhibition was not far short of that required for normal multiplication. Those eyes had received two doses of HN2, one on the first and one on the sixth day of the experiment.

If no further doses of HN2 are administered, recovery from the mitotic inhibition occurs in 3–6 days more and, by the 17th day, eyes removed from histological study showed basal cells of normal size and number though the full normal thickness of the five layers of epithelial cells had not yet been reached.

HEALING OF WOUNDS IN THE CORNEAL EPITHELIUM

Studies previously published from this laboratory (3) have shown that the healing of small pin prick wounds or cuts in the corneal epithelium takes place by migration of the adjacent cells into the denuded area without requiring any cellular multiplication during the actual period of wound closure. Indeed, mitotic activity is actually inhibited for several hours in the corneal epithelium surrounding a pin prick injury and can be suppressed over the entire cornea during the whole period of primary wound closure if a sufficient number of pin pricks are distributed over the surface. It was of interest to see whether this migratory activity was impaired by exposure to HN2.

Experiments were performed at low and at high doses and at various times after exposure to HN2. In all these experiments the rate of healing was found to be normal. It is of particular interest that the healing of pin prick wounds was normal even with doses of HN2 sufficient to produce marked loosening of the corneal epithelium. Fig. 3 shows a partially healed wound under such conditions. The corneal epithelium is so much loosened from the stroma that on fixation it has become detached and slightly folded in the neighborhood of the wound.

Even larger areas of corneal denudation are covered at a normal rate in spite of the absence of mitoses. This is perhaps not as paradoxical as it would at first appear for the corneal epithelial cells can in such instances become greatly flattened, each cell thus covering a much larger area than it normally would. Moreover, coverage of the defect can occur not only by migration into the denuded area of the remaining corneal epithelial cells. Conjunctival epithelium can

also participate in the covering of the defect. Indeed, with larger doses of mustard or nitrogen mustard large portions of the loosened corneal epithelium commonly slough off and the denuded area is often first covered by a migration of conjunctival epithelium into the defect. In pigmented animals whose limbal epithelial cells contain melanin pigment the movement of this line of pigment can often be readily seen. Fig. 4 shows a section through such a pigment line with epithelium of corneal type on one side of the pigmented cells, of con-

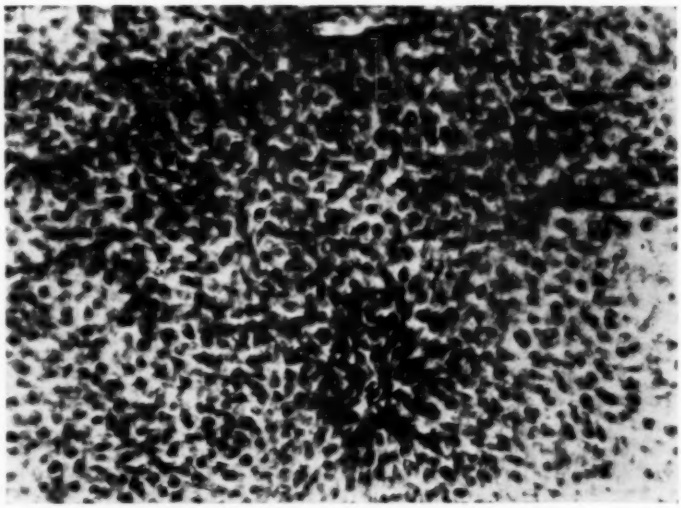

FIG. 3. Microphotograph of a healing pin prick in the corneal epithelium after treatment with nitrogen mustard. The folds in the corneal epithelium are evidence that loss of cohesion has occurred. Nevertheless, the wound healing is normal.

junctival type on the other side. The participation of conjunctival epithelium in the healing of corneal epithelial defects has been discussed in the preceding paper.

SYSTEMIC POISONING WITH HN2

It was of interest to know whether inhibition of mitosis in the cornea could occur if the poison was administered systemically and to compare the mitotic rates in other organs after systemic poisoning with those in the cornea.

Eight rats were used for the following experiment: two received a subcutaneous injection of 1.9 mg. per kilo of HN2 hydrochloride; two received the same dose of HN2 hydrochloride followed 24 hours later by 2 mg. per kilo of colchicine; two received colchicine and no HN2 hydrochloride; two were untreated controls. The animals were sacrificed 8 hours after the colchicine injections, 32 hours after the HN2 hydrochloride injections. Mitosis counts were made on cornea, bone marrow, and small intestine. The results in Table III show that HN2 hydrochloride injected intramuscularly produces a marked

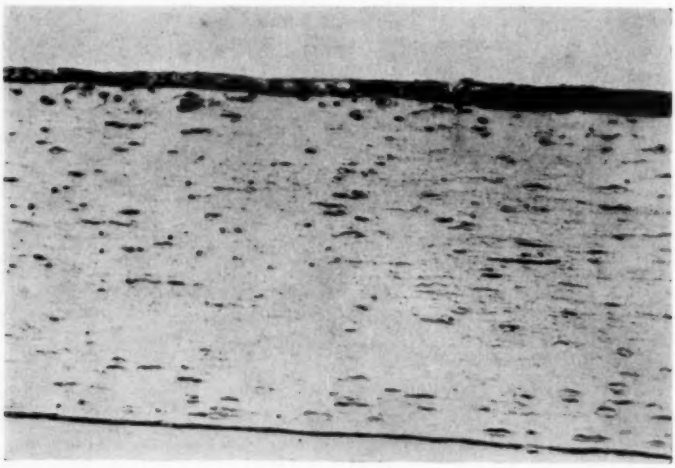

FIG. 4. OVERGROWTH OF CONJUNCTIVAL TYPE OF EPITHELIUM ON THE CORNEA AFTER EXPOSURE TO NITROGEN MUSTARD

inhibition of mitotic activity in cornea, bone marrow, intestine. With dosage used, the effect is most marked on the bone marrow, least marked on the cornea. This may mean that the cornea is more resistant to the effects of HN2 hydrochloride than is the bone marrow, but there may be some question as to whether the same concentration of the agent reaches both tissues after intramuscular injection.

This experiment indicates that inhibition of mitosis in the bone marrow and intestine constitutes a part at least of the effects of HN2

hydrochloride leading to delayed death. With the dosage used, it certainly does not account for the whole effect for, as has been shown by others, this dose leads after a somewhat longer interval to an extremely widespread depletion of bone marrow cells. If we assume that roughly half of the cells in the normal rat's bone marrow are cells which have completed the mitotic phases of their development and are merely maturing before subsequent passage into the blood stream, we cannot account for a disappearance of much more than one-half of all bone marrow cells solely by inhibition of mitosis. Other workers,

TABLE III

Inhibition of Mitosis by HN2 Hydrochlorid Injected Intramuscularly

	NORMAL CONTROL	HN2	COLCHICINE	HN2 AND COLCHICINE
Cornea Average number of mitoses per section (6 sections per cornea)	1.4	1.1	62.0	16.5
Intestine Average number of mitoses per crypts (12 crypts in cross section counted)	0.65	0.2	4.15	0.55
Bone Marrow % cell in mitosis	2%	too few to count	40%	too few to count

however, have found that 90% or more of the bone marrow cells disappear following such a dose as was used in this experiment and these lost cells cannot be accounted for by the appearance of immature cells in the blood stream. Moreover, such aplastic bone marrows are loaded with the debris of dead disintegrating cells. It must be concluded, therefore, that a dose of HN2 hydrochloride which leads to extreme aplasia of the bone marrow involves injury to resting cells in the bone marrow as well as inhibition of mitosis.

SUMMARY

Mustard and the nitrogen mustards are powerful inhibitors of mitosis in the corneal epithelium. The inhibition of mitosis can be produced by doses of these agents far smaller than those required to

produce clinically recognizable signs and symptoms of damage. With threshold doses the inhibition of mitosis comes on slowly, reaching a maximum many hours after the primary reaction of the toxic agent with the tissue has been completed. Recovery from the inhibition occurs spontaneously. The duration of the inhibition increases with the dosage applied and can be made to last for several weeks by repeated instillations of the poison. During a prolonged inhibition of mitosis the basal cells of the corneal epithelium increase in size. Wounds in the corneal epithelium heal at a normal rate even in the absence of mitotic activity. Cells that are in mitosis at the time of exposure to the toxic agents complete their division normally and at normal speed unless the dose applied is very large, under which circumstances some slowing down of the whole mitotic cycle occurs. Systemic administration of the poisons in approximately MLD 50 dosage causes an inhibition of mitosis in the corneal epithelium and also in the bone marrow and intestinal mucosa. Inhibition of mitosis represents the lowest threshold effect so far recognized in the reaction of tissues to these poisons.

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V. NUCLEAR FRAGMENTATION PRODUCED BY MUSTARD AND NITROGEN MUSTARDS IN THE CORNEAL EPITHELIUM*

JONAS S. FRIEDENWALD AND WILHELM BUSCHKE

In the preceding paper mention was made of a peculiar mode of death of some cells in the corneal epithelium after exposure to mustard or nitrogen mustard. The threshold dose at which this phenomenon first makes its appearance is 10–20 times that for mitosis inhibition. As will appear from the more detailed description below this is a form of karyorrhexis, a mode of cellular death that has been described in other tissues in a variety of conditions. We have preferred in this paper to call the phenomenon that we were observing by the non-committal descriptive term of nuclear fragmentation, avoiding thereby any implication that the mode of development and nature of this particular pathological process are similar to those of other instances of karyorrhexis that we have not investigated. Similarly disintegrating cells have been noted by others in the bone marrow and the other organs in systemic mustard poisoning. The present study was undertaken in the hope of throwing light on this peculiar pathological process and of determining whether and in what way the phemenon was related to mitotic activity.

I. DISTRIBUTION AND FREQUENCY OF NUCLEAR FRAGMENTATION

Isolated cells showing nuclear fragmentation are occasionally seen in the corneal epithelium of normal rats. Very extensive and numerous cell changes of this type are seen after exposure to ultraviolet light. In colchicine poisoning, in which mitosis is arrested in metaphase, dispersal of the chromatin can occur if the exposure to colchicine is prolonged. The final picture of such nuclear fragmentation under colchicine resembles the final picture after mustard and ultraviolet exposures, but, as will be noted below, the cytological steps by which the nuclear fragmentation is reached are not the same.

*The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

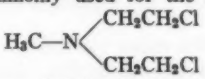
After instillation into rats' eyes of one drop of .03% or more of HN2[1] hydrochloride, cells in nuclear fragmentation are frequently seen. Owing to the frequent extracellular scattering of the nuclear fragments only very rough estimates of the number of affected cells can be given. They are found only in the basal layers of the epithelium[2] and usually about .04–1.0% of the basal cells show this change, although under special conditions, as will be noted below, the number of affected cells can be considerably increased. Even if the dosage of HN2 is increased to 1% or more, sufficient to produce complete destruction of the eye, the percent of cells showing this type of death is not appreciably increased though cells undergoing pycnosis appear in increasing numbers. Moreover the incidence of nuclear fragmentation following the same dose of HN2 varies markedly from animal to animal. If the eyes are enucleated, dipped for 10 minutes in solutions of .00125 to 0.03% HN2 hydrochloride in M/1000 HCl, and then incubated in moist chambers, nuclear fragmentation appears with much greater regularity and in a much larger number of cells.

These experiments made it clear that in the corneal epithelium only cells in a particular physiological state are susceptible to this kind of injury. It may be pointed out that in the corneal epithelium only cells in the basal layers undergo mitosis and that the number of mitosing cells normally found is of the same order of magnitude (0.4%) as the number which can be thrown into nuclear fragmentation by exposure to HN2.

II. CYTOLOGICAL STUDIES

By using the supravital technique with which nuclear fragmentation is regularly produced in abundance it is possible to obtain material in which the cytological development of the process may be studied. The first visible alteration is seen one to two hours after exposure, at which time scattered cells are found in which the nuclear chromatin shows a fine granular appearance. As time goes on the granules

[1] HN2 is the symbol commonly used for the nitrogen mustard



[2] With very prolonged supravital incubation similar changes also occur in other cell layers.

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V. NUCLEAR FRAGMENTATION PRODUCED BY MUSTARD AND NITROGEN MUSTARDS IN THE CORNEAL EPITHELIUM*

JONAS S. FRIEDENWALD AND WILHELM BUSCHKE

In the preceding paper mention was made of a peculiar mode of death of some cells in the corneal epithelium after exposure to mustard or nitrogen mustard. The threshold dose at which this phenomenon first makes its appearance is 10–20 times that for mitosis inhibition. As will appear from the more detailed description below this is a form of karyorrhexis, a mode of cellular death that has been described in other tissues in a variety of conditions. We have preferred in this paper to call the phenomenon that we were observing by the non-committal descriptive term of nuclear fragmentation, avoiding thereby any implication that the mode of development and nature of this particular pathological process are similar to those of other instances of karyorrhexis that we have not investigated. Similarly disintegrating cells have been noted by others in the bone marrow and the other organs in systemic mustard poisoning. The present study was undertaken in the hope of throwing light on this peculiar pathological process and of determining whether and in what way the phemenon was related to mitotic activity.

I. DISTRIBUTION AND FREQUENCY OF NUCLEAR FRAGMENTATION

Isolated cells showing nuclear fragmentation are occasionally seen in the corneal epithelium of normal rats. Very extensive and numerous cell changes of this type are seen after exposure to ultraviolet light. In colchicine poisoning, in which mitosis is arrested in metaphase, dispersal of the chromatin can occur if the exposure to colchicine is prolonged. The final picture of such nuclear fragmentation under colchicine resembles the final picture after mustard and ultraviolet exposures, but, as will be noted below, the cytological steps by which the nuclear fragmentation is reached are not the same.

*The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

After instillation into rats' eyes of one drop of .03% or more of HN2[1] hydrochloride, cells in nuclear fragmentation are frequently seen. Owing to the frequent extracellular scattering of the nuclear fragments only very rough estimates of the number of affected cells can be given. They are found only in the basal layers of the epithelium[2] and usually about .04–1.0% of the basal cells show this change, although under special conditions, as will be noted below, the number of affected cells can be considerably increased. Even if the dosage of HN2 is increased to 1% or more, sufficient to produce complete destruction of the eye, the percent of cells showing this type of death is not appreciably increased though cells undergoing pycnosis appear in increasing numbers. Moreover the incidence of nuclear fragmentation following the same dose of HN2 varies markedly from animal to animal. If the eyes are enucleated, dipped for 10 minutes in solutions of .00125 to 0.03% HN2 hydrochloride in M/1000 HCl, and then incubated in moist chambers, nuclear fragmentation appears with much greater regularity and in a much larger number of cells.

These experiments made it clear that in the corneal epithelium only cells in a particular physiological state are susceptible to this kind of injury. It may be pointed out that in the corneal epithelium only cells in the basal layers undergo mitosis and that the number of mitosing cells normally found is of the same order of magnitude (0.4%) as the number which can be thrown into nuclear fragmentation by exposure to HN2.

II. CYTOLOGICAL STUDIES

By using the supravital technique with which nuclear fragmentation is regularly produced in abundance it is possible to obtain material in which the cytological development of the process may be studied. The first visible alteration is seen one to two hours after exposure, at which time scattered cells are found in which the nuclear chromatin shows a fine granular appearance. As time goes on the granules

[1] HN2 is the symbol commonly used for the nitrogen mustard

$$H_3C{-}N\begin{matrix}\diagup CH_2CH_2Cl \\ \diagdown CH_2CH_2Cl\end{matrix}$$

[2] With very prolonged supravital incubation similar changes also occur in other cell layers.

become larger and fewer in number, and tend to accumulate on the nuclear membrane leaving a clear homogenous non-basophilic material in the nuclear space between them. At this stage many of the affected nuclei appear somewhat larger and rounder than normal (Fig. 1). About four hours after exposure the chromatin in some of the affected cells has all become aggregated into a few lumps and in some of the affected cells the nuclear membrane has disappeared. At this stage the appearance of the cell is much like that of normal mitosis in

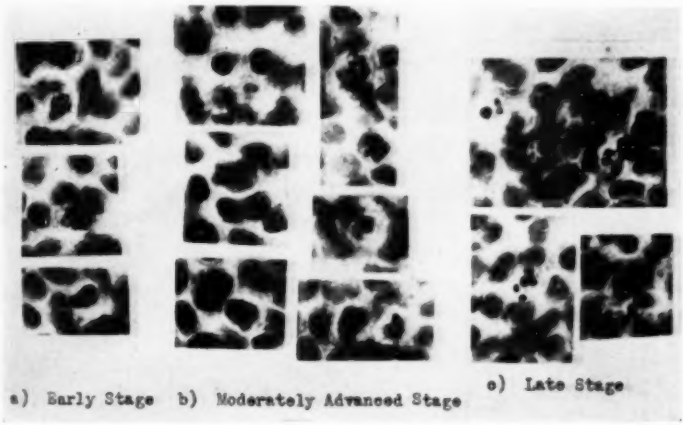

FIG. 1. NUCLEAR FRAGMENTATION IN THE RAT'S CORNEAL EPITHELIUM FOLLOWING EXPOSURE TO NITROGEN MUSTARD

metaphase with the chromatin lying in a zone of clear material in the center of the cell, only instead of chromatin being arranged in an orderly pattern of chromosomes, it is aggregated in irregular amorphous masses and no spindle is formed. The aggregated chromatin is Feulgen positive and the amount of Feulgen positive material in these cells is greater than in normal resting cells. Not all exposed cells reach this stage simultaneously, even at 4–6 hours after exposure one sees many cells in the prophase-like stage of chromatin aggregation within a still preserved nuclear membrane.

After the disappearance of the nuclear membrane the picture departs more and more from that of a normal mitosis. No spindle is formed. We have not been able to demonstrate regularly centrioles or astral

bodies in normal mitoses in the corneal epithelium, and can therefore attach no significance to the fact that these are also not seen in the process of nuclear fragmentation. With time the clear zone of nuclear material in the center of the cells disappears and the fragments of chromatin become dispersed throughout the cell body. Some cells in this stage appear swollen, others shrunken. Eventually the cell wall is ruptured and the cell debris discharged.

From the morphological point of view the process which leads to nuclear fragmentation can be described as a pathological and incompleted mitosis. Previous studies have shown that in the corneal epithelium the time required for a normally mitosing cell to pass through prophase and enter metaphase is one half hour or less (1). The pathological mitosis after exposure to HN2 requires upwards of two hours to proceed through comparable phases. There are evidently close analogies between the phenomenon that we are studying here and the prolongation of mitosis in invertebrate eggs found by Cannan (2) and coworkers after exposure to HN2.

III. EXPERIMENTS ON THE INHIBITION OR ARREST OF NUCLEAR FRAGMENTATION

Nuclear fragmentation following exposure to HN2 is prevented or delayed by incubation of the tissue at temperatures lower than the normal body temperature (Fig. 2). Quantitative studies on the nuclear fragmentation produced by exposure to ultraviolet light, reported elsewhere (3), show that the temperature coefficient for the development of this phenomenon is very large ($Q_{10} = 3.5$). A similar temperature coefficient was found for nuclear fragmentation produced by HN2.

Nuclear fragmentation following exposure to HN2 is prevented or delayed by anoxia. Nuclear fragmentation following exposure to ultraviolet light is likewise suppressed by anoxia.

In a series of experiments freshly enucleated rats eyes were dipped for 10 minutes in HN2 hydrochloride, freshly dissolved in isotonic phosphate buffer (pH 7.4), and then briefly washed in normal saline solution. Following this the eyes were placed in an incubator at 37°C, some in a moist chamber, some in aerated phosphate buffer, and some returned to freshly prepared HN2 solution which was aerated. After

5 hours of incubation they were fixed, and stained. Those incubated in the moist chamber showed the usual number of fragmented nuclei. Those in the phosphate buffer with or without the added HN2 showed no nuclear fragmentation. These experiments suggest that some factors necessary for the fragmentation process are removed in the bathing fluid.

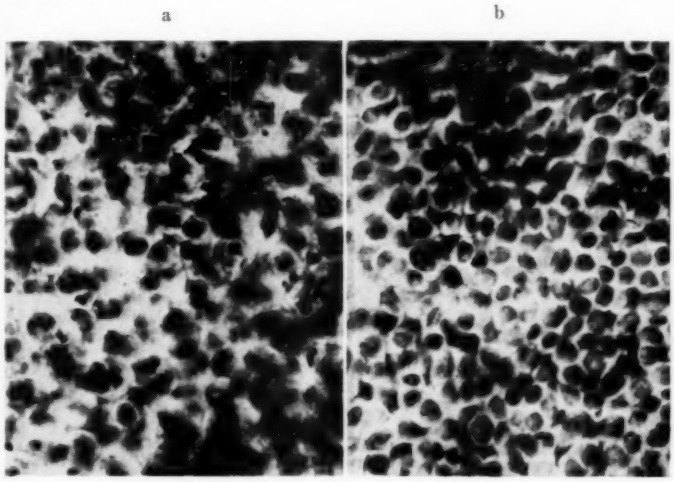

FIG. 2. CORNEAL EPITHELIUM AFTER EXPOSURE TO NITROGEN MUSTARD
a) Incubated at 37°C.
b) Incubated at 10°C.

It is concluded that the process of nuclear fragmentation involves certain aspects of the normal metabolic processes of the cells,—oxygen, normal incubation temperature, and a substance or substances extractable on washing the tissue. Whether the oxidative reaction concerned in this pathological process consists in the oxidation of some substrates not normally accessible to the respiratory chain of reactions, or whether some normally counterbalancing reductive process is inhibited can not be determined. As will be seen from the studies on corneal metabolism that are presented in later papers of this series, Dr. Herrmann has found no measurable disturbance in the overall metabolism of the tissue after exposure to doses of mustard

which produce only nuclear fragmentation and mitosis inhibition. The metabolic disturbance which is related to this effect is, therefore, a highly specific one involving only a small fraction of the total metabolic activity of the tissue. It may be noted, however, that low temperature, anoxia, and incubation of the tissue in aerated solutions also inhibits mitotic activity. It may be, therefore, that the inhibition of nuclear fragmentation by these agents operates through an inhibition of some factors necessary for normal mitosis, but no such positive conclusion is justified from experiments with these highly unspecific inhibiting procedures.

V. RELATION OF NUCLEAR FRAGMENTATION TO NORMAL MITOSIS

The question of the locus of injury which leads to nuclear fragmentation may be approached from several points of view. One may ask what part of the cell is injured or what cellular process is inhibited. One may ask at what phase of the cycle leading to cell division is the cell susceptible to this special type of injury. Finally, one may ask at what stage in the cycle of cell division does the process begin to deviate from the normal. All three of these questions are closely interlinked and have to be approached piecemeal, and only incomplete answers can be reached at the present stage of the study.

a) Effect of HN2 on cells in mitosis. The question of whether nuclear fragmentation results from an injury by HN2 to cells in the process of mitosis at the time of exposure is readily approachable. A variety of agents has been found:—adrenalin, ether anesthesia trauma, morphine, which prevent the entrance of cells into mitosis but do not interfere with the progress of cells that have already entered mitosis (1, 4). After administration of one of these agents the number of mitoses found in the cornea steadily declines to zero. First prophases disappear, then metaphases, etc., until about an hour after the onset of the inhibition no mitoses remain in the tissue. If the tissue is exposed to HN2 before or simultaneously with one of these mitosis inhibitors and samples are examined at various intervals after exposure, it is possible to determine whether the exposure to HN2 has altered the progress of cells through mitosis. Experiments of this type, similar to those reported in the previous paper, revealed

a slight slowing in the progress of cells through the mitotic cycle, but all normal mitoses disappear before any cells in the earliest recognizable state of nuclear fragmentation become visible. Those cells that are in mitosis at the time of exposure complete the cycle in an essentially normal though slightly delayed manner.

The reverse experiment can also be performed. The rat's cornea can be depleted of mitoses by a previous administration of an inhibiting agent, e.g., adrenalin, kept free of normal mitoses for the whole duration of the experiment, and HN2 administered. In such experiments, nuclear fragmentation develops following HN2 more regularly and more abundantly than when HN2 is given to control rats. It must be concluded that nuclear fragmentation does not develop from injury by HN2 to cells already in mitosis at the time of administration. The locus of the injury which leads to nuclear fragmentation, and the locus at which the pathological process diverges from the normal, is antecedent to the histological visible stages of mitosis.

b) Pre-Mitosis. The nature of this pre-mitotic susceptible state can be defined more specifically as follows: Cells which once have reached the histologically recognizable states of mitosis are apparently irreversibly committed to the mitotic process. We may inquire whether the locus of HN2 injury which leads to nuclear fragmentation lies before or after the irreversible entry of the cell into the mitotic process. Evidence on this point can be obtained from an analysis of the inhibition of mitosis produced by adrenalin and other similarly acting agents. When adrenalin is injected intramuscularly into a rat, there results a period during which no new mitoses appear in the corneal epithelium; depending on the dosage the duration of this period varies. By using a suspension of adrenalin in peanut oil the inhibition can be made to last up to 10–12 hours. With the waning of the adrenalin effect mitoses reappear and during the first hours of this recovery period the number of mitoses commonly overshoots the normal level. The overshooting, however, is small and brief and, in our experience, never is sufficient to make good the previous deficit, nor does the overshooting increase with the prolongation of the inhibition. It follows that the locus of inhibitory action of adrenalin on the mitotic cycle is not later than the point of irreversible entry into mitotic activity,

for, if the inhibition acted only at some later point in the cycle, cells would accumulate at the normal rate in the preinhibited state and would escape from this state in the recovery phase, with a resulting excess overshooting of mitosis numbers that should compensate for the deficit accumulated during the period of inhibition.

Since HN2 produces nuclear fragmentation even when an adrenalin inhibition of entrance into normal mitosis exists at the time of HN2 administration and throughout the whole period of development of the nuclear fragmentation, we can conclude that the locus of action of HN2 is antecedent to the irreversible entrance of cells into mitosis. This antecedent state cannot be the normal resting state since only a very few cells in the cornea are at any given moment susceptible to this form of injury. It may be suggested, therefore, that there exists a reversible pre-mitotic state antecedent to the irreversible mitotic state, and that in the corneal epithelium this pre-mitotic state is the source of the cells which after exposure to HN2 undergo nuclear fragmentation.

In order to study this relation quantitatively, experiments were performed in which one drop of 0.25% HN2 hydrochloride was instilled into one each of a series of rats at various times before or after the intramuscular injection of 0.2 mg. of adrenalin in peanut oil. The eyes were removed for histological study 6 hours after the administration of HN2. The animals in these experiments were kept each in a separate cage in the quietest part of the laboratory and were not handled or disturbed for at least 12 hours before the beginning of the experiment. This was done in order to reduce to a minimum the excitement of the rats, and to avoid adrenalemia not caused by the injections. The control rats in this series which received no adrenalin injection, and also those that received the adrenalin simultaneously with, or later than, the HN2, showed minimal numbers of cells with nuclear fragmentation. When HN2 was given 1 to 4 hours after the adrenalin injection relatively great numbers of cells in nuclear fragmentation resulted. Adrenalin alone never causes nuclear fragmentation. Similar results can also be obtained if the inhibition of mitosis is produced by a hypodermic injection of morphine. Under morphine hypnosis the body temperature of rats is markedly lowered. Since decreased temperature had already been shown to delay greatly

the onset of nuclear fragmentation, the animals in this experiment were kept warm in the incubator.

These experiments show that when the normal entry of cells into irreversible mitosis is blocked then some cells accumulate in the pre-mitotic state. This is not in contradiction with the previous conclusion that the susceptible pre-mitotic state is reversible. If cells in the pre-mitotic state normally can escape from this condition either by entering mitosis or by returning to the resting state, then the closing of one of the exits will, other things being equal, lead to an accumulation of cells in the pre-mitotic state until the rate of escape through the remaining exit is equal to the rate of entrance. This is, in fact, in accord with the phenomenon of overshooting regularly seen in the recovery phase after adrenalin inhibition. This conclusion is not unequivocal since, obviously, adrenalin and morphine might increase the number of cells in the susceptible state through some action wholly unrelated to their effects on the mitotic process.

Since in the control animals with minimal adrenalemia the number of fragmented nuclei produced by HN2 is very small, we can conclude that there are few cells in the susceptible state under these conditions, and hence that the normal duration of the susceptible state in the corneal epithelium is quite short in comparison to the whole inter-mitotic period. Not all procedures which lead to an inhibition of mitosis lead to an increased susceptibility to nuclear fragmentation. For instance, if eyes are enucleated and placed in a moist chamber in the incubator, mitoses appear in decreasing numbers during the second hour of incubation, and by the end of three hours and for several hours thereafter no mitoses are visible in the tissue. If, at the end of three hours incubation the eyes are exposed to HN2 and then re-incubated, nuclear fragmentation does not develop. Evidently some factors necessary for the mitotic process and also factors necessary for the fragmentation process are exhausted by this procedure.

Effect of Hypotonicity and Low pH

If freshly enucleated rats eyes are dipped for 10 minutes in a freshly prepared 0.25% solution of HN2 in M/6 phosphate buffer, and then incubated in a moist chamber for 6 hours, only a moderate number of epithelial cells develop nuclear fragmentation, the number being

comparable to that produced in vivo. On the other hand, if the enucleated eyes are dipped in HN2 hydrochloride solution dissolved in M/1000 HCl and then incubated, the number of cells developing nuclear fragmentation is very much greater. A variety of experiments were performed in attempting to elucidate this peculiar finding. If the HN2 is dissolved in 0.9% NaCl containing M/1000 HCl, fewer nuclear fragmentations are produced than in the hypotonic M/1000 HCl solution without NaCl, but more than are produced by exposure to HN2 in the phosphate buffer. If the eyes are dipped into distilled water or M/1000 HCl solution before being placed for 10 minutes in a solution of HN2 in the phosphate buffer, there is a moderate increase in the number of nuclear fragmentations produced. Apparently both hypotonicity and low pH before or during the exposure favor the development of the lesion.

The interpretation of these findings is complicated by the fact that HN2 is transformed more slowly into its reactive derivative, the cyclic ethylene imine, at low pH and in the presence of high concentrations of chloride or phosphate ions, but, since the intracellular ionic changes are probably small compared with those in the bathing fluid, it seems likely that hypotonicity and low pH produce their effect by increasing the number of susceptible cells rather than by radically altering the reactivity of the toxic agent. In this connection it is to be remembered that similar brief exposures to abnormal salt concentrations and acidities have been used by many investigators to precipitate parthenogenesis in various ova.

V. LOCUS OF MITOSIS INHIBITION BY HN2

As previously reported, the administration of very small doses of HN2 to rats' eyes results in a slowly declining mitotic activity without the production of nuclear fragmentation. With appropriate dosage (one drop of .004–.008% HN2 hydrochloride) mitoses disappear completely from the corneal epithelium in 18 hours, reappear in about 36 hours, and overshoot the normal numbers about 48 hours following exposure. In a series of experiments a second dose of more concentrated HN2 hydrochloride solution was administered either at the period of mitotic inhibition or at the period of mitosis excess. For controls the opposite eyes of the same animals were used and these

received either only the first small dose of HN2 or only the second large dose of HN2. Six hours after administration of the second dose the eyes were enucleated. It was found that the first small dose caused no nuclear fragmentation and that it resulted neither in an appreciable increase nor in an appreciable decrease of the number of fragmented cells following the second dose. It follows: (1) that the locus of mitosis inhibition by HN2 cannot be antecedent in the mitotic process to the locus of injury producing nuclear fragmentation; and (2) that the locus of mitosis inhibition by HN2 must be different from that by adrenalin and morphine. Consequently the mitosis inhibition must be either at the same pre-mitotic stage as that in which larger doses lead to nuclear fragmentation, or it must be at some stage in the irreversible portion of the mitotic cycle, i.e. at a later stage than the locus of adrenalin inhibition. The former of these two alternatives would seem a priori more likely, and the latter can be exluded by direct experimental test, for if the mitosis inhibition of HN2 occurs at a later stage in the mitotic cycle than the inhibition of adrenalin, then the overshooting of mitosis numbers during the recovery from HN2 inhibition should be relatively refractory to adrenalin inhibition. Experiments revealed that mitotic activity during the recovery phase following HN2 inhibition is just as susceptible to adrenalin inhibition as is mitotic activity normally. The conclusion that the locus of mitotic inhibition by HN2 is the pre-mitotic state is in agreement with the findings of Cannan on invertebrate eggs.

DISCUSSION

The course of events in the normal cycle which is indicated by this analysis may be represented schematically as follows provisionally identifying the susceptible state with that of pre-mitosis:—

$$\text{Resting State} \underset{\text{B}}{\overset{\text{A}}{\rightleftharpoons}} \text{Susceptible State} \xrightarrow{\text{C}} \text{Mitosis}$$

Adrenalin, morphine, cause an inhibition at (C) leading to an accumulation of cells in the susceptible state. Cells in this condition when exposed to larger doses of HN2 are damaged in such a way that after some hours they undergo a pathological process leading to nuclear fragmentation. Even with smaller doses of HN2 progress

through the pre-mitotic state toward normal mitosis is inhibited. A simple hypothesis that is compatible with all the data so far obtained, is that in the larger doses HN2 inhibits the return of cells from the pre-mitotic state to the resting state. Such an hypothesis presupposes that the pre-mitotic state is essentially unstable, and that cells frozen in this state, unable either to return to the resting state or to escape into normal mitosis proceed through such portions of mitotic activity as they are still capable of.

The notion of an intrinsic instability of the pre-mitotic state receives some support by analogy from the experiments of Loeb (5) on parthenogenesis which have been confirmed and extended by many other investigators. These experiments show that cells exposed to agents which stimulate parthenogenesis tend to undergo cytolysis. If they are protected from cytolysis by what Loeb called "compensating agents" parthenogenesis results. This analogy must not be pressed too far for the cytolysis which has been observed in these experiments is a very different process from that of nuclear fragmentation. Special susceptibility of cells in pre-mitosis to X-ray injury has been suggested by Strangeways and Oakley (6), and our own experiments do not exclude the possibility that cells in this state are directly damaged by the HN2, not merely frozen in an unstable state.

If susceptibility to nuclear fragmentation on exposure to HN2 can be taken as a sign that a cell is in the pre-mitotic state, then the number of cells normally present in this state in the corneal epithelium is small when the animals are in basal condition (no adrenalemia), roughly one tenth the number normally present in the histologically recognizable state of mitosis. Assuming that at least as many cells enter this state per hour as enter mitosis it can be concluded that the normal duration of the pre-mitotic state is about one tenth the duration of normal mitosis, i.e., no more than 5–10 minutes. Again judging from the maximum number of cells which show fragmentation when HN2 is administered after various inhibitors of mitosis one can conclude that cells may stay in the premitotic state for an hour or two under these conditions.

The analysis of our experiments furnishes an explanation for the fact that rapidly growing tissues are susceptible to injury by mustard. It has previously been supposed that cells in mitosis are particularly

susceptible. Our studies on the influence of various agents on cells already in mitosis show them to be remarkably resistant. The pre-mitotic state, on the other hand, is highly vulnerable to some agents.

The visceral lesions of systemic mustard poisoning appear to concern primarily those tissues in which the mitosis rate is high. It would seem likely that many of these lesions could be interpreted as the results of pathological mitosis and nuclear fragmentation. Certainly the changes in the bone marrow suggest such an interpretation. To some degree, therefore, tissue susceptibility to mustard may be related to the frequency of cells in pre-mitosis in different tissues.

In the foregoing discussion we have provisionally identified the susceptible state in the corneal epithelium with the state of premitosis. The reasons for suggesting this identity are: (1) Nuclear fragmentation due to mustard occurs in the corneal epithelium almost exclusively in the normally mitosing layer. (2) The morphological steps through which nuclear fragmentation develops bear certain analogies to the prophase of normal mitoses and are associated with an increase in the Feulgen positive material in the chromatin. (3) Various non-specific processes which inhibit the mitotic process,—low temperature, anoxia, washing the tissue in inert solutions,—also prevent the development of nuclear fragmentation. (4) Certain drugs which, without causing major damage to the tissue, inhibit the visible onset of mitosis and facilitate the accumulation of cells in the susceptible state. (5) Some other tissues, (e.g. bone marrow) with a high mitotic rate, are particularly susceptible to injury by mustard and show evidence of a similar mode of cellular death.

While the arguments listed above are cogent, though not conclusive, in respect to the phenomena we have studied in the corneal epithelium, it must be definitely stated that this argument cannot be generally extended to other tissues. Certain exceptions can already be noted.

The endothelium of the cornea has a low rate of mitosis, in fact we have never seen a mitosis in this tissue though mitotic divisions may occur during regeneration following injuries. Nevertheless, after exposure to adequate doses of mustard or HN2, the cells of the corneal endothelium in the exposed area uniformly show nuclear fragmentation. The same uniform susceptibility to this type of death is shown by wandering cells, both monocytes and polymorphonuclear

leucocytes, which are normally present in the corneal stroma of animals. The similarity of the process in the corneal endothelium and in the wandering cells in the corneal stroma to that which we have been studying in the corneal epithelium is further shown by the fact that anaerobic incubation suppresses the lesion in all three cell types. Thus the state of susceptibility to nuclear fragmentation following exposure to the mustards cannot, in the corneal endothelium at least, be identified with the state of premitosis. To do so would require the wholly unwarranted assumption that these normally not mitosing cells are constantly in a state of premitosis.

Moreover, not all actively mitosing cells are susceptible to this type of injury. By partial hepatectomy it is readily possible to provoke a high rate of mitosis in the remaining liver tissues. We have performed experiments in which, during the period of maximal mitotic activity following partial hepatectomy in a rat, a MLD 50 dose of HN2 was injected intramuscularly. The animals were sacrificed 24 hours later. Their bone marrows showed extensive evidence of nuclear fragmentation but the regenerating liver was free from any such lesion. It follows that the liver cells, even when actively multiplying are not specially susceptible to this type of injury.

Finally it must be pointed out that ultraviolet radiation produces in the corneal epithelium large numbers of cells with nuclear fragmentation, the morphological characteristics of which closely resemble those produced by the mustards, but after exposure to ultraviolet radiation it is the more superficial layer of not normally mitosing cells which show this lesion while the basal cells largely escape.

It follows from all this that while the pathological process which leads to nuclear fragmentation involves some aspects, for instance the elaboration of Feulgen positive material, which are normally a part of the mitotic process, and while, in the corneal epithelium susceptibility to this type of injury by the mustard agents is suggestively connected with a state of premitosis, the identification of the susceptible state with that of premitosis holds neither for some other tissues nor for some other injurious agents.

In the preceding article it was pointed out that nuclear fragmentation occurs in the corneal epithelium with doses of mustard or nitrogen mustard insufficient to produce clinically recognizable signs of damage

in the rat's eye. It should be remembered, however, that in human beings pain, photophobia, lachrymation, and fine punctate stippling of the corneal surface are produced by doses of these toxic agents much smaller than those required to produce gross opacification of the cornea, loosening of the epithelium and purulent conjunctival discharge. These latter signs of a severe necrotizing lesion were the ones on which we had to depend for the recognition of clinically evident damage in the rat's eye. It would seem likely, therefore, that the irritative symptoms of sub-necrotizing doses in human beings are associated with the occurrence of nuclear fragmentation in the epithelial cells. Since more direct evidence of the connection between nuclear fragmentation and irritative symptoms is available in respect to ultraviolet light burns of the eye, this point will not be argued further here.

SUMMARY

1. Nuclear fragmentation is a mode of cellular death induced in the corneal epithelium by threshold doses of mustard and nitrogen mustard. Most of the experiments reported here were performed with HN2, but similar results were obtained with mustard and other nitrogen mustards. When the toxic agent is applied in vivo, only cells in the basal layers exhibit this type of reaction and only a small percent of the basal cells are affected, the numbers and location of the affected cells being similar in order of magnitude to those normally found in mitosis. Increase in the dose of the toxic agent does not increase the number of cells showing this mode of death beyond a certain low limit.

2. The cytological changes involved in the development of nuclear fragmentation are remarkably similar to those of prophase and metaphase in normal mitosis. It is suggested that the phenomenon of nuclear fragmentation in these experiments may be a form of pathological mitosis.

3. The development of nuclear fragmentation after exposure to mustard is inhibited by lowered temperature and by anoxia. Both of these experimental conditions likewise inhibit progress through the normal mitotic cycle. Immersion of the tissue in Ringer's fluid or phosphate buffer suppresses the nuclear fragmentation, presumably

by extracting some necessary substances from the tissue. Similar immersion likewise suppresses mitosis in this tissue. Supravital maintenance in a warm moist chamber for three hours exhausts the capacity of the tissue to produce new mitoses. After such supravital maintenance, exposure to HN2 fails to produce nuclear fragmentation.

4. On the basis of experiments reported, it is postulated that the passage of cells from the resting state through mitosis involves two steps: (a) a state of excitation (pre-mitosis) from which they can enter mitosis or return to the resting state; (b) actual mitosis which, once begun, cannot be readily reversed. Experiments with mustard and nitrogen mustard applied to the cornea, under conditions of mitosis inhibition by various other agents, demonstrates that nuclear fragmentation does not result from injury by mustard to cells already in mitosis, but that it is probably the cells in pre-mitosis which, on exposure to mustard, undergo nuclear fragmentation. The pre-mitotic state is not the sole and sufficient condition for susceptibility to nuclear fragmentation from exposure to mustard. There are some tissues, for instance the corneal endothelium, whose cells show a uniform susceptibility to this type of injury in spite of a very low rate of mitosis. There are other tissues, for instance the liver, whose cells show a high resistance to this type of injury in spite of a high rate of mitosis induced by partial hepatectomy. On the other hand, the bone marrow and intestinal mucosa show both a high susceptibility to nuclear fragmentation and a high rate of mitosis.

5. A simple hypothesis that will explain the present findings is that cells in pre-mitosis are, after exposure to mustard, unable either to enter normal mitosis or to return to the resting state, and that after being held in the pre-mitotic state unduly long they proceed into a pathological mitosis which ends in nuclear fragmentation. This hypothesis presupposes that the pre-mitotic state is an unstable one in which the cell cannot long maintain its equilibrium. An alternative hypothesis is that exposure to mustard directly damages cells in the pre-mitotic state and thus induces the pathological process leading to nuclear fragmentation.

6. Brief exposure of the tissue to hypotonic solution, to solutions of low pH, and particularly to HN2 dissolved in hypotonic solutions of low pH, greatly increase the number of cells susceptible to this injury.

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VI. NOTE ON KARYOLYSIS OF THE CORNEAL STROMA CELLS*

JONAS S. FRIEDENWALD

It was noted in the preceding paper that wandering cells normally present in the corneal stroma of rats' and beef eyes undergo nuclear fragmentation upon exposure to mustard and nitrogen mustard, but that the stroma cells, sometimes called corneal corpuscles or keratocytes, do not show this mode of death. With adequate dosage the stroma cell nuclei swell, become pale staining with margination of the chromatin, and eventually burst (Figs. 1–4). Evidence of this type of change can be seen in ordinary histological sections as noted in one of the preceding papers (1). These phenomena are seen only at dosage levels that cause easily recognizable clouding of the cornea and often cause permanent corneal scarring. Similar modes of death of the corneal stroma cells are seen with a wide variety of injurious agents,—arsenicals, alkali burns, freezing, etc.

The phenomenon can be observed in enucleated eyes kept in a moist chamber in the incubator, in which case infiltration with inflammatory cells is avoided and the histological picture rendered more simple, and is most readily observed in flat sections of the tissue or in flat preparations such as were used in the previous paper. For the latter purpose the tissue, after fixation, is soaked for one hour in a saturated aqueous solution of amyl alcohol. After removal from this solution the corneal epithelium and endothelium are found to be markedly loosened and can readily be wiped off, yielding a clean preparation of the corneal stroma which can be stained, cleared, and mounted without sectioning. Most of the experiments reported in this paper were performed on rats. If only the cornea is incubated in a moist chamber neither swelling nor bursting of the nuclei is observed. If corneas, previously exposed to mustard or nitrogen mustard, are incubated not in a moist chamber but in physiologic saline solution, the stroma nuclei swell but do not burst. If normal corneas with uninjured epithelium and

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

FIG. 1

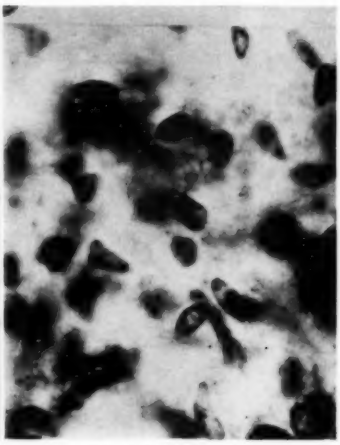

FIG. 2

FIG. 1. Stroma cells of rat's cornea incubated 7 hours in phosphate buffer (control). The denuded corneal stroma was fixed, stained, and cleared without sectioning.

FIG. 2. Stroma cells of rat's cornea incubated 9 hours in phosphate buffer after exposure to nitrogen mustard. Same magnification as Figure 1.

FIG. 3

FIG. 4

FIG. 3. Same as Fig. 2, incubated for 12 hours showing bursting nuclei.

FIG. 4. Same as Fig. 2, incubated for 16 hours. Only nuclear debris remains.

endothelium are incubated in saline very little swelling of the stroma nuclei takes place, but if normal corneal stroma denuded of endothelium and epithelium is incubated in saline the stroma nuclei swell but do not burst.

These observations lead to the conclusion that extra fluid must enter the corneal stroma in order to enable the stroma nuclei to swell. As long as the epithelial and endothelial barriers remain normal, the normal state of detergescence of the corneal stroma is maintained even supravitally (2). If, however, these barriers are injured then swelling of the nuclei can take place. All of the agents, which in our experience cause swelling of the stroma nuclei, cause a loosening and eventual sloughing of the epithelium and endothelium. However, normal stroma nuclei in the presence of available fluid swell but do not burst. Consequently, after exposure to mustard and other similarly injurious agents the fragility of the nuclear boundary is greater than normal and the nuclei burst. The fact that this bursting of the nuclei occurs in vivo or on incubation of the whole eye, but not on incubation of the isolated cornea, indicates that the ocular tissues or fluids provide some factor, possibly a metabolite, necessary for this pathological process.

It was of interest to discover whether the increased fragility of the stroma nuclei was an immediate or delayed effect of the toxic injury. Experiments were performed in which the corneal endothelium in enucleated eyes was injured mechanically and the eyes then kept for 16 hours in a refrigerator. At the end of this period samples were examined and the stroma nuclei were found swollen but not bursting. Some of these eyes were then exposed to HN2 hydrochlorid and incubated in a moist chamber at 37°C. Samples removed for histologic study at varying intervals showed that the bursting of the swollen stroma cell nuclei occurred about 10 hours after exposure to the HN2, that is at about the same time following exposure as in experiments in which there was no corneal oedema at the time of exposure.

Nuclear fragmentation and karyolysis are not the only modes of cellular death following exposure to mustard. With very large doses of mustard or nitrogen mustard pycnosis of all types of corneal cells is observed, increasing in frequency with increasing dosage.

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VII. THE ADHESION OF EPITHELIUM TO STROMA IN THE CORNEA*

HEINZ HERRMANN AND FAY H. HICKMAN

The advantages of the cornea as a test object for the study of cellular physiology are demonstrated by investigations in many fields. The geometrical simplicity of its structure and the toughness of its connective tissue stroma render it almost uniquely favorable for the study of certain aspects of cellular cohesion. The cohesion of cells and tissues is one of the fundamental factors in the biological organization. However, the phenomena concerned with cellular cohesion have been the subject of only a few experimental studies. Fenn (1) measured the adhesiveness of leucocytes to various solid surfaces, such as glass and carbon. Fenn (2) and several other authors (3, 4) have attempted to relate the magnitude of the adhesive forces to the decrease of the surface tension.

Our experiments deal with the cohesion of the basal layer of the epithelium to the stroma of the cornea. The bulk of the stroma is made up of collagen fibres which give to the stroma its great mechanical strength. The stroma cells lie scattered between the collagen bundles and are entirely absent in the most superficial layers of the stroma. In several species the epithelium is separated from the stroma by a distinct membrane known as Bowman's membrane, but in some species this membrane is absent. In the beef corneas, which were used in our experiments, no morphologically distinct Bowman's membrane is present.

An epithelium supported by and adherent to a fibrous stroma is a very common pattern of histological organization. In most cases the epithelium and the stroma form a morphologically complex aggregate, as for example the tubules in the kidney, the acini in various glands, and the villi in the intestine. In the cornea the separating boundary between stroma and epithelium forms a smooth plane of simple configuration. This simplicity of the boundary and the leath-

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

ery consistency of the stroma make it possible to remove the epithelium quantitatively and measure the adhesive forces under different experimental conditions.

TECHNIQUE

a. *Measurement of Adhesion.* Under certain experimental and pathological conditions the adhesion of the epithelium to the stroma

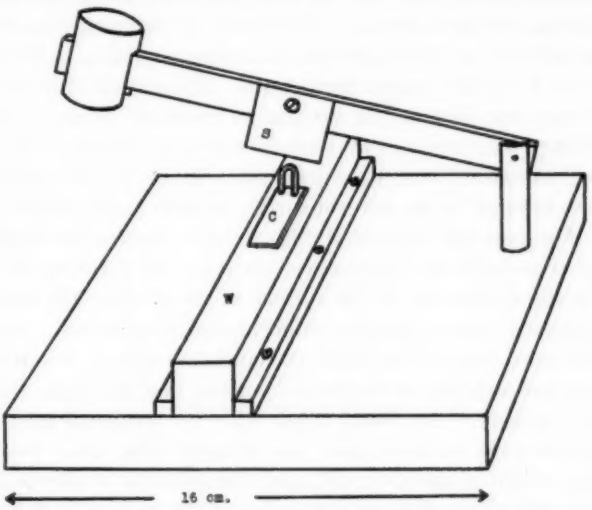

FIG. 1. SCRAPER FOR TESTING THE ADHESION OF THE CORNEAL EPITHELIUM
s. scraper blade
c. corneal strip
w. sliding wood block

is so greatly reduced that the epithelium can be wiped off with great ease, and may, in fact, be displaced by a gentle blink of the lids. In order to place this qualitative observation on a more objective and quantitative basis a simple mechanical scraper was used.

The scraper (Fig. 1) consists of a blade (S) of rustless steel attached to a lever arm. The blade is 0.2 mm. thick and the scraping edge is bevelled at an angle of about 70°. By lowering the lever arm the blade can be brought to rest on a corneal strip (C) secured by a staple

to a smooth wooden block (W). The pressure exerted on the cornea by the blade can be varied by changing the position of a weight on the lever arm to which the scraper is attached. A brass cylinder weighing 99.5 gm. was used as a weight. The force with which the blade rests on the cornea was determined by means of a balance for the unweighted lever and for 5 standard positions of the weight. The pressures used were 40, 60, 95, 110, 165, 200 gm. respectively.

Corneal strips, about 8 mm. in width, were excised along the greatest diameter of beef corneas. The width of the corneal strips were kept as uniform as possible in order to avoid variation in the bearing surface on which the scraper blade rests. The corneal strip which was to be tested was fastened at one end to the wood block by means of an ordinary wire staple, and straightened and flattened out with a glass rod moistened with physiologic salt solution. The scraper blade was then lowered to the corneal surface as near to the staple as possible. The bevelled edge of the blade faces toward the staple; the vertical edge faces the free corneal surface. By thrusting the blade through the epithelium to the surface of the stroma, the epithelium can be incised and an upright epithelial edge established. The blade was then once more lifted above the epithelial surface, the weight on the lever arm adjusted to the desired position, and the blade reinserted into the incision. The wood block with the attached strip of the cornea was then pushed under the scraper (Fig. 1a). Depending upon the weight of the lever and upon the adhesion of the epithelium, the blade will either glide over the edge of the incision in the epithelium and slide over the surface, or the epithelium will be pushed off in front of the blade, which then moves along the surface of the stroma. The speed with which the wood block is pushed under the blade is not critical within wide limits. We completed one stroke in approximately one half second. In removing the epithelium with the scraper the separation generally takes place at the boundary between stroma and epithelium and not between the epithelial layers themselves. The removal of very small areas of epithelium can readily be recognized since the glossy appearance of the epithelium is replaced by the dull stroma surface.

The epithelium which has been removed with the scraper can be collected without difficulty. Any epithelium which remains adherent

to the corneal strip is taken off with a sharp scalpel. By comparing the amount of epithelium removed by the scraper with that subsequently removed by the scalpel, the percent removed by the scraper may be calculated, thus rendering the measurements independent of the total area of the strip that is used.

The amounts of epithelium removed by scalpel or scraper were measured in most experiments by determining the protein nitrogen of the samples by the micro Kjeldahl method. In one series of experiments in which we tested the effect of a great number of substances over a wide range of concentrations, an approximate esti-

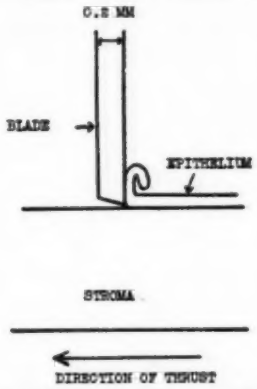

FIG. 1a MODE OF OPERATION OF SCRAPER

mate was made by determining visually whether more or less than one half of the epithelium had been removed from the strip at a given pressure. This procedure was very efficient, and, when used with the practice obtained in several hundred tests, it was sufficiently accurate for the particular purpose.

With a moderate constant weight on the scraper (90, 110 gm.) successive strokes remove approximately the same amount of epithelium until the supply is almost exhausted (Fig. 2). With variable pressure and a fixed number of strokes, the amount of epithelium removed is not a linear function of the pressure. A critical region is found in which a small change in pressure causes a large change in the amount removed. We found that with a weight on the scraper of

less than 90 grams very little epithelium is removed in the standard procedure. With a scraping weight of 200 gms. a large part of the epithelium is removed (Fig. 3). When the adhesion of the epithelium to the stroma is decreased, under certain experimental conditions, the critical range of the scraping weight which causes the disruption of the cohesive forces is found at a lower range than in the normal controls. By measuring the per cent of epithelium removed with different scraping weights the range of this critical zone can be determined.

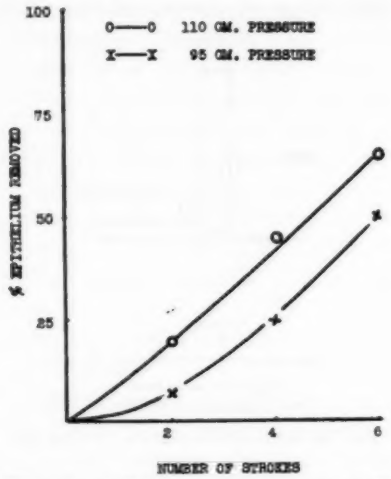

FIG. 2 PERCENT OF EPITHELIUM REMOVED WITH INCREASING NUMBER OF STROKES

b. *Manipulation and maintenance of the cornea.* Beef eyes obtained from the slaughter house arrived at the laboratory not longer than three hours after the death of the animal.[1] Each eye was washed shortly with running lukewarm tapwater. Eyes with opacities, cuts, or other defects on the cornea were discarded.

Some tests were conducted by immersing the excised cornea directly in 25–50 ml. of various solutions.[2] The controls in these tests were

[1] We are deeply indebted to the management and employees of Schluderberg-Kurdle Co., meat packers who supplied us with the beef eyes and who spared no effort to enable us to obtain the material in the freshest possible state.

[2] If not indicated otherwise all solutions were adjusted to a pH of 7.2–7.4.

immersed in NaCl solutions of about the same ionic strength. This type of experiment was used with substances available in considerable amounts and with which rapid effects were of interest (e.g. various detergents and ions). Substances available only in smaller quantities, or the effect of which had to be tested after longer time intervals, were injected into the stroma. Routinely the injection was carried out while the cornea was still attached to the eyeball. A 1 ml. tuberculin syringe was used with a No. 26 or 27 needle, which was introduced near the limbus at 4–6 symmetrically placed points of

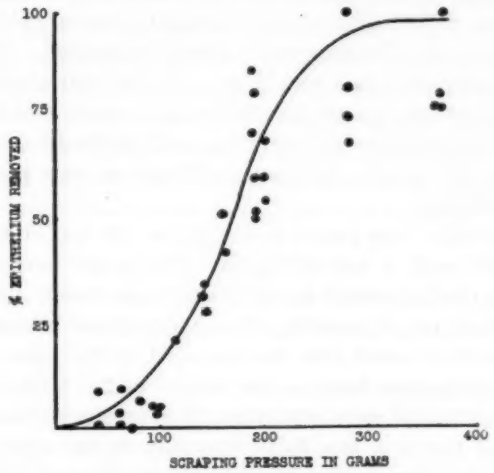

FIG. 3 PERCENT OF EPITHELIUM REMOVED WITH TWO STROKES AND VARIABLE LOAD

the corneal circumference. The point of the needle was forced gently almost to the center of the cornea. The fluid was injected slowly with a minimum necessary pressure and was thus deposited in several more or less confluent areas covering the greater part of the cornea. The total fluid volume injected per cornea was 0.25 ml. About $\frac{1}{2}$–$\frac{1}{3}$ is lost by drainage, and in assays of the metabolic utilization of injection material the actual amount remaining has to be determined in a sufficient number of controls. We found that 0.45% NaCl solutions, and even distilled water, are well tolerated as solvent for the injected substances. The adhesion of the epithelium is not affected

by injection of 0.25 ml. of such solutions, and the slight bulging and opacity of corneas which follows the injection disappears almost completely on subsequent incubation. A smooth surface is of importance for a satisfactory test with the scraper.

In case a longer maintenance was desired the injected corneas were excised and washed by dipping them three times into a beaker of physiologic saline. The excess fluid was drained off, and the corneas were placed on filter paper (epithelium upwards) for a more complete drainage of excess fluid, and covered with a petri dish for protection. Adherent drops of fluid were removed by cautiously touching the rim of the cornea with a filter paper. Contact between epithelium and filter paper to any greater extent should be avoided. The corneas were then transferred to a watchglass with the epithelium upwards, trapping, if possible, an air bubble, so as to avoid contact between the corneal endothelium and the glass, and maintaining the natural convexity of the tissue. As many as 30 corneas were placed on one 16 cm. watchglass.

The watch glass was placed in a glass jar (20 cm. in diameter, 20 cm. in height) with a well fitting lid. The proper humidity can be obtained by placing several layers of wet paper towels under the lid. At a proper degree of humidity the corneas should neither be dehydrated to such an extent that the rims stick to the glass, nor should water of condensation form on the surface of the epithelium. This latter precaution is of great importance for a satisfactory maintenance. We observed that any free fluid, especially on the upper epithelial surface of the cornea, greatly increases the incidence of bacterial contamination, which is the main danger to the successful maintenance during a longer incubation period. Fortunately very small bacterial colonies can be detected as little gray spots on the transparent tissue. Infected corneas were discarded. The resistance of the uninjected cornea to bacterial contamination must be considerable. Working without special precautions of sterility, infection was rarely observed even after incubation for 20 hours at 32°C. and it was extremely rare at an incubation temperature of 29°C. The incidence of infection was somewhat more frequent with injected corneas. However, the use of sterile instruments, sterile wash fluid and glassware, and face masks during the preparation of the cornea decreased the incidence of infection.

The adhesion of the epithelium in uninjected corneas remains practically unaltered during incubation at 29°C. for 20 hours. Increase of the incubation temperature above 35°C. decreases the adhesion slightly and promotes the development of bacterial colonies. The adhesion of the epithelium in corneas with injected fluid in the stroma is more affected by incubation. In about one third of the samples the critical pressure drops to 80–100 grams. However, even in these samples the amount of epithelium removed at 40 grams is always less than 35%. It is advisable to use rather large numbers of corneas in each experiment, and to determine the effect of the tested substances not with a single dose but preferably by defining the highest ineffective and the lowest effective concentration, and to carry along a sufficient number of controls. The controls are injected with a saline solution of approximately the same tonicity as the solution injected into the experimental samples. If such precautions cannot be applied, an effect should not be regarded as significant unless more than 70% of the epithelium is removed at a pressure of 40 gms.

RESULTS

With the method described in the preceding paragraphs, we tested the influence of various experimental factors upon the adhesion of the epithelium.

1. *Temperature.* The corneas were exposed for different lengths of time to a wide range of temperatures, and the effect was tested immediately after termination of the exposure, and also after a subsequent incubation period of 20 hours at 29°C.

The effect of higher temperatures was tested by placing the corneas in warmed solutions of 0.9% NaCl. At 52°C. the tissue becomes hazy and the epithelium is rapidly loosened. Within ten minutes the critical scraping pressure drops to below 40 grams. Exposure to 45°C. for half an hour produced no immediate effect and none was observed on subsequent incubation at 29°C. However, continuous exposure to 45°C. for many hours produces a gradual loosening of the epithelium.

For the exposure to low temperatures, the corneas were put into petri dishes. No fluid was added but the dishes were covered with wet paper towels in order to maintain adequate humidity. The petri dishes were kept for the desired length of time in the main

compartment of a refrigerator at −2°C. or in a freezing unit at −15°C. After exposure for one hour to −2°C. no effect could be noticed. After freezing of the corneas at −15°C. and subsequent thawing, the critical scraping pressure was found to be normal, but with subsequent incubation for 10 hours at 29°C. marked loosening of the epithelium developed (Table I).

2. *Hydrogen Ion Concentration and salts.* Corneas were bathed for one hour in physiologic saline, the pH of which was adjusted (color indicator) to a desired value by addition of N/1 HCl or NaOH. Glycine buffer of equal molarity was used instead of saline for the establishment of pH 11 and 12. Three to five corneas were used for each test. The pH of the solutions shifts slightly within the first

TABLE I

Effect of Temperature on Adhesion of the Epithelium

TEMPERATURE IN °C.	TESTED IMMEDIATELY		TESTED AFTER INCUBATION AT 29°C. FOR 20 HOURS	
	Pressure in grams	% Epithelium removed	Pressure in grams	% Epithelium removed
52° for 10 min.	40	98, 96		
45° for 30 min.	95	30	95	20, 25
−2° for 60 min.	95	21	95	20, 49
−15° for 15 min.	95	35	40	98
−15° for 30 min.	95	28	40	95

half hour due to the buffer capacity of the corneas and has to be readjusted. After one hour the corneas were removed from the bath, drained briefly and tested with the scraper.

Over a wide range of the pH scale no significant effect was observed (Table II). In strongly acid and alkaline solutions much epithelium was removed at a pressure of 95 grams, but these results were due to its complete disintegration rather than to a removal at the boundary. At pH 12 the epithelium is transformed into viscous strands, and at pH 1 only the superficial portion of the epithelium was removed leaving a considerable extent of the stromal surface covered by the basal layer. The disintegration of the epithelium at low pH seemed to be determined not only by the concentration of hydrogen ions, but also by the ionic strength as such, for we observed regularly that the

epithelium came off as a whole at a pressure of 40 grams when the N/10 solution of HCl was made up with distilled water (Table III) instead of saline. The adhesion was normal or even slightly greater than normal when the hydrochloric acid in the bathing fluid was replaced by acids which are characterized by their protein precipitating capacity (5, 6).

TABLE II

Influence of pH on Adhesion of the Epithelium

pH	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
1.0	95	55, 67, 62
2.0	95	29, 44, 33
3.5	95	42, 22, 36
4.5	95	11, 14
6.3	95	18
7.0	95	20
9.0	95	15, 12
11.0	95	91
	60	43, 38
12.0	60	77, 80

TABLE III

The Effect of Various Protein Precipitating Acids on the Adhesion of the Epithelium

REAGENTS USED 0.1 M.	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
Tannic Acid	165	12
	200	66
Sulfosalicylic Acid	165	42
Trichloracetic Acid	165	48
Metaphosphoric Acid	200	53
Tungstic Acid	165	59

Neutral solutions of most of the salts tested were without significant effect. Salts of the alkaline earth caused some decrease of the adhesion, but the effect could be recognized only if the excess of these ions were removed by a prolonged washing of the corneas in saline. Thiocyanate and urea were also somewhat effective (Table IV). Removal of calcium either by prolonged washing with saline or by suspending the corneas in N/5 oxalate solutions for one hour did not alter the adhesion (Table V).

3. *Alcohols.* A series of aliphatic alcohols was compared by bathing the cornea for one hour in solutions of these compounds in 0.9% saline. The members of this series with the longer carbon chains were far more effective than the homologues with a small number of carbon atoms. Polyalcohols such as glycerol and sucrose were ineffective. In fact, on adding glycerol to butyl alcohol, the loosening

TABLE IV

Effect of Solutions of Various Substances on the Adhesion of the Epithelium

Corneas placed for 1 hour in M/1 solution at pH 7

INEFFECTIVE		LOSS OF ADHESION
Sodium Chloride	Sodium Sulfate	Calcium Chloride
Potassium Chloride	Sodium Bisulfite	Barium Chloride
Potassium Iodide	Sodium Thiosulfate	Strontium Chloride
Sodium Fluoride	Sodium Nitrate	Potassium Thiocyanate
Potassium Bromide	Disodium Phosphate	Urea
Sodium Acetate	Mono Sodium Phosphate	
Glucose	Ferric Chloride	
Sodium Citrate	Magnesium Sulfate	
Sodium Oxalate	Copper Sulfate	
Glycerol	Lanthanum Nitrate	

TABLE V

Adhesion of the Epithelium after Bathing of the Corneas for 1 hour in Solutions of Various Ions

REAGENTS USED	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
Calcium Chloride, 1.0 M, pH 6.5	95	95
	40	51
Lithium Sulfate, 1 M, pH 6.8	95	21, 9
Sodium Oxalate, 0.2 M, pH 6.8	95	11, 13

effect of the latter could not be detected if tested immediately after the treatment. However, this antagonistic action disappeared, and a complete loss of adhesion occurred if the samples were washed with saline and the excess of glycerol was removed. Glycerol was ineffective as an antagonist if it was applied, not simultaneously, but subsequently to the treatment with butyl alcohol (Table VI).

4. *Butyl derivatives.* In view of the efficacy of butyl alcohol, we tested some other derivatives of the butyl radical. Butyric acid and butylamine in 1M concentration are ineffective at a pH of 6.5. How-

TABLE VI

Effect of Alcohols on Adhesion of the Epithelium

REAGENT USED	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
Methyl alcohol 10 M	95	18
Ethyl alcohol		
10 M	40	65
5 M	95	63
2.5 M	95	24
Peopyl alcohol		
5 M	40	88
2.5 M	40	81
1.2 M	95	25
Butyl alcohol		
0.5 M	40	98
0.25 M	95	15
0.12 M	95	11
Amyl alcohol		
0.2 M	40	92
0.1 M	95	17
Glycerol 1 M	95	16
Glycerol 1 M + Butyl alcohol 0.5 M (tested immediately after treatment)	95	43, 34
Glycerol 1 M + Butyl alcohol 0.5 M (tested after washing in saline for 1 hour)	40	95, 87

ever, at pH 2 butyric acid is somewhat more effective than the alcohol, and at pH 4.5 it has a slight effect. Butyl amine is ineffective up to pH 8.5. At still higher pH butyl amine becomes insoluble.

An enhancement of the efficacy of butyl alcohol by the introduction of a chlorine atom is seen in comparing butanol with chlorobutanol, and ethanol with ethylene chlorhydrin. Another related compound, chloral hydrate, is somewhat less effective as a loosening agent.

The ether of butyl alcohol with ethylene- or diethylene glycol is as effective as butyl alcohol. Polyglycols themselves up to a chain length of nine carbons have no loosening effect but, on the contrary, they seem to increase the cohesion of the tissue.

5. *Detergents.* Butyl alcohol and the derivatives tested contain both hydrophilic and hydrophobic groups assymetrically arranged in the same molecule. We have extended our tests to compounds in which this assymetry is even more pronounced. Such substances are generally classified as detergents. Mannitan laurate was used as as example of a neutral detergent. The hydrophilic group in this compound is a poly-alcohol. In concentrations up to 1M it had no effect on the adhesion of the epithelium to the stroma.

In the anionic detergents which we used, the hydrophilic group is a sulfate or sulfonate radical. These substances do not loosen the epithelium from the stroma but gradually transform the epithelium into a slimy mass. The process starts in the superficial layers of the epithelium. If it is interrupted at an early stage, the basal layer is found still intact and firmly adherent. This phenomenon seemed unrelated to that which we were studying and was not investigated further. The lowest concentration at which this effect was observable was found to be between 0.1 and 0.01 M.

In the cationic detergents the hydrophilic group is a quaternary ammonium or pyridinium radical. Three of the four substances in this group which we tested caused a loosening of the epithelium. With the substances the effect was somewhat different from that produced by butyl alcohol. The epithelium became hazy and fragile and did not peel off in a coherent sheet. With the lower concentrations the effect seemed to be confined to the superficial layer of cells. The lowest effective concentration was about 0.005M (Table VII).

6. *Enzymes.* Solutions of the various enzymes were injected into beef corneas and the tissues incubated at 23–24°C. for 4 hours or more, after which the adhesion of the epithelium to the stroma was tested. Recrystallized samples of trypsin and chymotrypsin were

kindly supplied by Dr. R. Herriott (Rockefeller Institute, Princeton). They were obtained in the form of ammonium and magnesium sulfate

TABLE VII

Effect of Various Substances on the Adhesion of the Corneal Epithelium

	LOWEST MOLAR CONC. EFFECTIVE IN LOOSENING EPITHELIUM	HIGHEST MOLAR CONC. WHICH FAILED TO LOOSEN EPITHELIUM
A. Alcohols and Related Compounds		
Butanol	0.5	0.2
Butyrate		
pH 2	0.3	0.1
pH 4.5	1.0	0.5
pH 6.5		1.0
Butylamine		
pH 7		1.0
pH 8.5		1.0
Chlorobutanol	0.06	0.03
Ethylenchlorhydrine	0.5	0.25
Chloralhydrate	1.2	0.8
Butylcellosolve	0.5	0.2
$CH_3(CH_2)_3OCH_2CH_2OH$		
Butylcarbitol	0.5	0.2
$CH_3(CH_2)_3O(CH_2)_2O(CH_2)_2OH$		
Diethyleneglycol		3.0
$OHCH_2CH_2OCH_2CH_2OH$		
Nonaethyleneglycol		3.0
$HO(CH_2CH_2O)_9H$		
B. Neutral Detergents		
Mannitan laurate		1.0
Mannitan lauric acid ester Atlas Co.		
C. Cationic Detergents		
Zephiran (Alba Pharmaceutical Co.)	0.005	0.002
Alkyl dimethyl benzyl ammonium chloride (alkyl = C_8—C_{18})		
Triton K-60 (Röhm and Haas)		0.2
Lauryl dimethyl benzyl ammonium chloride		
Emulsol 607	0.005	0.002
Lauric acid ester of colamino-formyl methyl pyridinium chloride		
Emulsol 607 M(Emulsol Co.)	0.005	0.002
Myristic acid ester of colaminoformyl methyl pyridinium chloride		

TABLE VII–*Continued*

	LOWEST EFFECTIVE CONCENTRATION PRODUCING LIQUEFACTION (MOLAR)	HIGHEST MOLAR CONC. WHICH FAILED TO LOOSEN EPITHELIUM
D. Anionic Detergents		
Duponel (E. I. duPont de Nemours) Sodium lauryl sulfate	0.005	
Drene (Proctor and Gamble) Triethanolamine lauryl sulfate	0.005	
Igepon AP (General Dyestuffs) $R—COO—(CH_2)_2SO_3Na$	0.005	
Triton W-30 (Röhm and Haas) Sodium salt of alkyl phenoxy ethyl sulfonate	0.005	
Tergitol-7 (Carbon and Carbide Corp.) $C_4H_9 \cdot CH \cdot (C_2H_4) \cdot C_2H_4 \cdot CH(SO_4Na)-C_2H_4CH(C_2H_5)_2$	0.005	
	LOWEST MOLAR CONC. EFFECTIVE IN LOOSENING EPITHELIUM	
E. Enzymes		
Trypsin	5–10 mg./cornea	0.5 mg./cornea
Chymotrypsin	5–10 mg./cornea	0.5 mg./cornea
Hyaluronidase		1 mg./cornea
Ribonuclease		1 mg./cornea
Pancreatic lipase		Activities see text

cakes. Solutions of these preparations were dialyzed against running tap water for 6 hours and then overnight in the icebox against 0.9% saline. The protein content was determined by precipitating the protein in an aliquot of the dialyzed sample with trichloracetic acid and determining the nitrogen content in the thoroughly washed precipitate. A recrystallized preparation of ribonuclease was also obtained from Dr. R. Herriott. A purified and highly active sample of hyaluronidase prepared from bull testis was supplied by Dr. K. Meyer, Columbia University. Pancreatic lipase was prepared and purified by Dr. M. Bovarnick (Department of Biochemistry, Johns Hopkins Medical School). The amounts of this preparation which were used for injection hydrolyzed triacetin equivalent to 0.7 ml. of N/10 NaOH and tributyrin equivalent to 3.9 ml. N/10 NaOH per hour at 37°C. The enzyme was free from proteolytic activity. From

Table VII it can be seen that among these enzymes only trypsin and chymotrypsin cause a decrease of the adhesion under our experimental conditions.

7. *Inhibition of metabolism.* The role of oxygen for the maintenance of the tissue cohesion was assayed by incubating the corneas under anaerobiosis. A desiccator was used as moist chamber for the preservation of the cornea. After placing the corneas inside, the vessel was evacuated for ten minutes to a pressure of 50 mm. Hg. Afterwards the desiccator jar was placed in an incubator and connected with a tank of nitrogen or a mixture of 95% N + 5% CO_2. The gases were purified by passing through an alkaline pyrogallol solution or over copper wire at 500°C. A gentle flow of the gas was maintained

TABLE VIII

Adhesion of the Epithelium after Injection of Cyanide and Malonate and Incubation at 29°C.

REAGENT INJECTED PER CORNEA pH 7.0	INCUBATION TIME HOURS	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
Sodium Cyanide	12	95	31
1 mg.	20		25
(approximately 0.02 M)			
5 mg.	12	95	12
(approximately 0.1 M)	20		15
Sodium malonate			
2 mg.	20	95	25, 35
(approximately 0.08 M)			

throughout the incubation period. After anaerobic incubation for 20 hours the corneas were perfectly clear and the epithelium showed the same adhesion as the controls. Incubation for 10 hours anaerobically and for the subsequent 10 hours aerobically was likewise without effect. The insensitivity of the adhesive mechanism to the availability of oxygen is demonstrated also by another experiment in which cyanide was injected in amounts up to 5 mg. per cornea, equivalent to a concentration of approximately 0.1 M. Again no sign of loosening was detectable, and malonate (which is supposed to inhibit succindehydrogenase) was also without influence (Table VIII).

On the other hand a great decrease of the adhesion can be observed after injection of iodoacetate and fluoride (Table IX and Fig. 4)

TABLE IX

Effect of Iodoacetate and Fluoride on the Adhesion of the Epithelium

REAGENTS INJECTED	INCUBATION AT 32°C. FOR 12 HOURS		INCUBATION AT 29°C. FOR 20 HOURS	
	Pressure in grams	% Epithelium removed	Pressure in grams	% Epithelium removed
Fluoride, 0.3 mg. per cornea (ap-	40	90	40	87, 92
(proximately 0.007 M)	40	50	40	49
0.1 mg.	60	76	60	61
	40	28, 20		
0.03 mg.	60	17	60	18, 37
Iodoacetate, 0.03 mg. per cornea (approximately 0.001 M)	40	96, 93	40	61
0.01 mg.	60	35	40	
	95	56	60	14
0.003 mg.	60	22	60	28
	95	31		

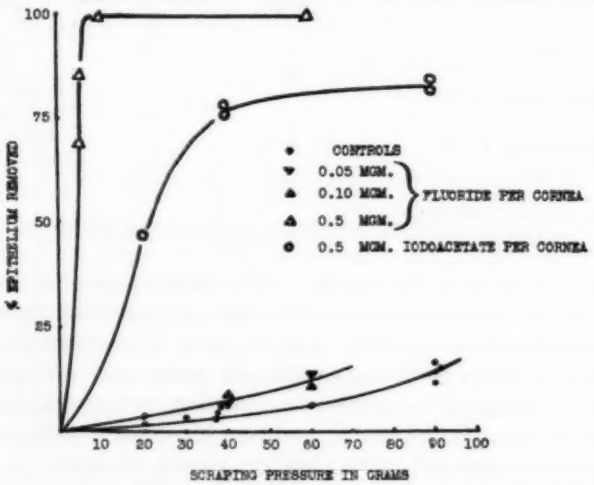

FIG. 4 EFFECT OF IODOACETATE AND FLUORIDE ON THE ADHESION OF THE CORNEAL EPITHELIUM

which are known primarily as inhibitors of reactions in the glycolytic pathway. The effect in respect to the loosening of the epithelium developed after a lag period of about 6–10 hours. At lower incubation temperatures the effect decreased markedly (Table X).

DISCUSSION

a. *Method:* Our method for the measurement of the adhesion of the epithelium of the corneas has been developed on a purely empirical basis. Closer analysis shows that the connection between our figures and the adhesive forces is an indirect one, as may be seen from the following simplified scheme of the process of separation.

Adhesion of epithelium to stroma is here defined as the force with which the epithelium resists mechanical removal from the stroma. For a direct determination of the adhesion it would be necessary to measure the forces which are required to pull the epithelium from the stroma. Such a force could be applied either in a direction vertical

TABLE X

The Effect of Incubation and Temperature upon the Loss of Adhesion Caused by the Injection of Fluoride and Iodoacetate

REAGENT INJECTED PER CORNEA	18°C.		29°C.	
	Pressure in grams	% Epithelium removed	Pressure in grams	% Epithelium removed
Sodium fluoride				
0.12 mg.	40	4, 17, 22	40	96, 87, 81
	60	21, 16		
	80	26		
0.25 mg.	40	33, 24	40	79, 80
Sodium Iodoacetate				
0.5 mg.	40	10, 33	40	73, 89
	60	19, 29	40	85, 84
	95	23, 57	40	88, 87

to the surface of the epithelium, or as a shearing force parallel to the stroma surface. As long as the adhesion of the epithelium is normal, or only moderately decreased, it is hardly possible to remove any epithelium by pulling vertically to the corneal surface. Only when the tissue has completely lost its cohesion can the epithelium be pulled off in large flakes or in one whole sheet, (e.g. after bathing in butyl alcohol or after incubation with higher doses of iodoacetate or fluoride). Therefore, only the second alternative seemed practicable. If the epithelial edge, towards which the pushing force of the scraper is directed, stands perpendicular to the surface of the stroma, the values obtained for this force should be a direct measure

of the adhesive forces (Fig. 5). Actually the contact angle between stroma and epithelium will be greater than 90°. In this case the force with which the epithelium will counteract the pressure of the scraper (R′) will be represented by two components (see Fig. 5). The one (*a*) counteracting the horizontal force of pressure, the other (*b*) acting on the blade in vertical direction. By splitting R′ into two components (*a* and *b*) two magnitudes should become available as indices of the adhesion. The possibility of measuring component (*a*) by determining the force which is necessary to pull the scraper

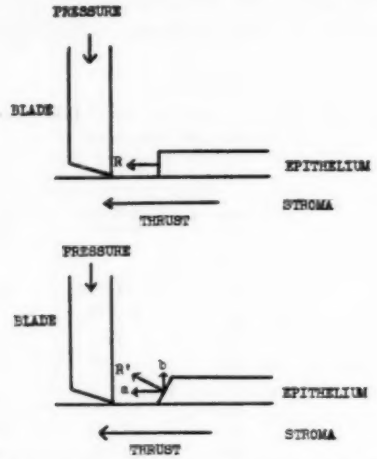

FIG. 5 FORCES INVOLVED IN SCRAPING OFF EPITHELIUM

over the surface of the stroma eliminating by a suitable weight the action of component (*b*) seems hardly feasible since the friction on the stroma surface would be of a comparable order of magnitude. In our procedure component (*b*) is measured by determining the weight which is required to keep the blade on the surface of the stroma while the epithelium is removed. An uncontrolled variable in determining component (*b*) is the contact angle between epithelium and stroma. This angle will depend upon the turgescence and general condition of the epithelial cells. Our practical experience shows, however, that these variables do not interfere with the results of our relatively

crude measurements. We observed the same difference in the adhesion between experimental samples and controls in direct measurements, and after fixation of the corneas in 5% trichloracetic acid, during which differences in the state of the epithelial cell and its turgescence should have been eliminated. We were unable to detect a difference in the water content (as index of turgescence) of control and experimental samples after iodoacetate injection which caused a complete loss of the adhesion of the epithelium. Although it appears that the differences in our measurements indicate actual differences in the cohesion of the tissues, turgescence and the other factors which influence the contact angle would have to be considered if smaller variations in the adhesion than those which are described in our experiments were to be measured.

b. *Results:* With our experiments we attempted to obtain some information about the physical or chemical basis of the adhesion of the epithelium to the stroma. The aggregation of single cells in multicellular organisms requires either the presence of an enveloping extracellular coat, or cohesion of the cells to one another. These two principles have been sharply contrasted in an extensive study by Holtfreter (7) who pointed out that the cells are bound together by an external coat at early stages of the embryonic development, whereas the cell surface proper becomes the site of cohesion at more advanced stages of differentiation. An instance where a more or less specific intercellular cement is supposed to provide the connecting element between cells is the capillary endothelium (8). Great significance has been attributed to this substance in relation to the fragility of the capillary wall.

The chemical qualities of the external coat are badly defined, and only a few of its properties have become known (9). It is greatly altered by a change of the pH from 6.4 to 8.0, and is stable only in the presence of calcium ions. Experiments with these agents failed to loosen the epithelium of the cornea. The removal of calcium by washing the cornea with oxalate solution has no noticeable influence, and the adhesion remains unchanged over the greater part of the pH scale. Therefore, it seems unlikely that the epithelium is attached to the stroma by a material which is similar to the external coat of developing eggs or to the intercellular cement of the capillary wall.

It would seem more likely that the adhesion in the case of the cornea epithelium is a property of the cell surface itself, or of the surface of the collagen bundles of the stroma.

The stroma has a very high content of a mucopolysaccharide and experiments were carried out to ascertain the role of this substance as a possible factor for the adhesion of the epithelium to the stroma. The mucopolysaccharide of the cornea has been isolated and analyzed by Meyer and Chaffee (10) who found it to contain one sulfo group per molecule. Sulfonated mucopolysaccharides are acids with great affinity to the positively charged groups of the protein molecule. At low pH these mucopolysaccharides form insoluble salts with proteins and these salts are not decomposed by comparable concentrations of anions with lower protein affinity, e.g. chloride (11). However, on addition of anions with greater or equal affinity an exchange reaction takes place, the characteristics of which are determined by the relative affinities and concentrations of the competing ions. On the alkaline side of the isoelectric point of the protein component the salts undergo dissociation.

As a first step, we attempted to test the possibility that such an acid base linkage across the stroma epithelium barrier might be the basis for the cohesion of the tissues. Our experiments, however, do not support this assumption. The following data are relevant in this connection. (a) In the alkaline range loosening of the epithelium does not occur below pH 10. In order to relate this finding to the hypothetical acid-base cohesion one would have to assume that adherent protein has its isoelectric point about pH 10. This is unlikely since only the proteins of nucleus are known to have this property. (b) If the hypothesis were correct, loosening of the epithelium should occur in the presence of anions with strong protein affinity. In contrast, we found that such ions cause a slight increase in the adhesion of the tissue. (c) Our only experimental finding which would seem to be in agreement with the above assumption is the loosening of the epithelium in the presence of high concentrations of calcium salts, since calcium ions have apparently a considerable dissolving action on mucopolysaccharides. However, calcium ions have so many affinities with tissue components that no specific interpretation can be offered to explain this finding. It should be pointed out that calcium

has, e.g., a high affinity to cephaline in monomolecular layers (12). (d) The failure to obtain any effect on epithelial adhesion by any other of the many ions we have tested also speaks against the hypothesis of an acid base linkage between stroma and epithelium. (e) The participation, in particular, of strongly ionized substances in the cornea, such as mucopolysaccharides or nucleic acids is made unlikely by the negative results with hyaluronidase and ribonuclease, the enzymes which are supposed to destroy these compounds.

These negative results are discussed in detail because the rejection of an ionic linkage as the basis of the cohesion compelled us to consider possible non-ionic forces. In experiments with alcohols the efficacy was found to increase with increasing length of their aliphatic chains. The effect of some alcohols is enhanced by the further development of the hydrophobic portion of the molecule, as for example in chlorine substituted compounds. Amplification of the hydrophilic group, on the other hand, does not enhance the effectiveness, in fact polyalcohols operate in the reverse direction, increasing the adhesion of the epithelium and suppressing the loosening effect of butanol if added simultaneously to the corneas.

These findings could be interpreted by assuming that a lipoid layer is of significance in the establishment of the adhesive mechanism. The presence of lipoids in the surface layer of cells is well established. A detailed picture of the molecular pattern of the cell surface has been proposed by Danielli and Harvey (13) and has been elaborated on by Danielli and Davson (14) and by Danielli (15). The experimental basis for the hypothesis of these authors was obtained by measurement of the surface tension of marine eggs and other monocellular organisms. According to these authors the cell surface can be described as a double layer of lipoid molecules, their hydrophobic tails oriented towards each other, and a layer of protein adsorbed by their hydrophilic heads. The structure of such a multilayer should be markedly disturbed by the addition of long chain alcohols. Thus, for example, it may be supposed that the alcohols are absorbed in the lipoid double layer as the result of the lipoid affinity of their hydrocarbon chains, while at the same time introducing an aqueous phase between the two sheets of the lipoid double layer. A possible representation of such a disruption can be found in a paper by Schmitt

(16). In suggesting this as a possible explanation for the loosening of the epithelium by alcohols we are assuming that the boundary between epithelium and stroma consists of a molecular array similar to that suggested by Danielli and co-workers for the individual cell of unicellular organisms. In connection with the antagonistic effect of glycerol and butyl alcohol on the loosening of the epithelium an interesting observation, mentioned in the discussion (p. 24) of an article by Parpart and Dziemian (17), should be referred to. These authors found that the penetration of glycerol into red blood cells of some species is accelerated by butyl alcohol and in other species suppressed. It should be pointed out that a disruption of a multilayer, such as is described above, could take place at different levels. In all cases the phenomenon of the loss of adhesion will appear the same, as long as no finer means of differentiation become available. Some evidence on this point can be seen in the fact that injection of a solution of recrystallized trypsin and chymotrypsin causes a loosening of the epithelium which indicates the presence of a protein in the coherent multilayer. An interpretation of the adhesion of the epidermis of the skin based entirely upon the properties of the collagen of the dermis has been advanced in a recent publication by Felsher (18).

Whatever the molecular arrangement which has to be regarded as the direct cause of the cohesion, it is remarkable that aerobiosis is not required for the maintenance of the adhesion of the epithelium, as demonstrated in experiments under anaerobiosis and after injection of cyanide. On the other hand the dependence on anaerobic metabolic processes is suggested by the sensitivity of the adhesion to iodoacetate and fluoride, two metabolic poisons known to interfere with the anaerobic phase of the carbohydrate metabolism. An analysis of the present status of our knowledge of the metabolism of the cornea will be given in one of the following papers. As an example of other cellular activities, involving the cell surface, which are maintained under anaerobiosis and impaired by iodoacetate and fluoride, mention should be made of the phagocytic activity of leucocytes as discussed in an article by Fleischmann (19, pp. 291–292).

c. *Relation to vesicants:* Loosening of the corneal epithelium is a phenomenon which invites comparison with that of vesication in the skin. In addition to the effects of various chemical and physical

agents reported in this paper it may be pointed out that bacterial contamination causes a loss of cohesion in the corneal tissues supravitally, and that infection and inflammation of the cornea lead to loosening of the epithelium in vivo. Loosening of the epithelium after exposure to ultraviolet light is a well known clinical phenomenon and has been observed experimentally (20). For the present, however, we wish merely to point out that the analogies are not complete.

Experiments by Danielli (21) on the vesication in frogs' skin in which the effects of various metabolic poisons were explored revealed some significant contrasts in comparison to our results in the cornea. For instance, cyanide and azide fail to loosen the corneal epithelium, but cause loosening of the epithelium in the frog's skin. On the other hand, iodoacetate is a powerful agent in loosening the corneal epithelium but was found ineffective on the frog's skin by Danielli. These differences may be due to differences in the species or in the tissues studied, and indicate that the metabolic pathways by which energy is supplied for the maintenance of similar aspects of tissue organization may differ in different tissues or in different species.

SUMMARY

1. A simple mechanical device is described for the quantitative estimation of the adhesion of the epithelium to the corneal stroma.

2. Exposure of the tissue to temperatures over 45°C. or freezing and subsequent incubation lead to a loosening of the corneal epithelium.

3. No loosening of the epithelium was observed on altering the pH over the range pH 3 to pH 9, on removal of calcium, on addition of protein precipitating agents, or on exposure to a wide variety of inorganic salts. Exceptional among the latter were sodium thiocyanate and high concentrations of calcium chloride, both of which caused loosening of the epithelium.

4. Exposure of the tissue to aqueous solutions of alcohols in low concentration markedly decreased the tissue cohesion. The minimum effective concentration decreased with increasing molecular weight of the alcohols, amyl and butyl alcohol and some chlorine substitutes of the latter were the most effective agents of this type so far tested.

On the other hand polyalcohols such as sucrose or glycerol failed to loosen the epithelium.

5. The cohesion of the tissue is well maintained on anaerobic incubation and in the presence of cyanide and of malonate. Loosening of the epithelium occurs on incubation of tissue exposed to iodoacetate or fluoride.

6. Exposure to proteolytic enzymes causes a loosening of the epithelium.

7. These studies reveal no evidence in favor of the presence in the cornea of an intercellular cement similar to that characteristic of the capillary wall. Furthermore, they indicate that the cohesive force is probably not due to electrostatic attractions of oppositely charged ionic groups. On the contrary, the experiments suggest that the adhesive forces originate in the attraction of non-polar hydrocarbon residues in the boundary surface, and that the coherent surface may be a protein lipoid multilayer. In attributing the adhesive force to hydrocarbon residues lipoids may be considered as the most likely source, but the affinities of non-polar side chains of proteins such as collagen are not excluded at the present. The maintenance of the coherent organization may require metabolic energy, since the cohesion is lost on iodoacetate and fluoride poisoning.

8. The phenomenon of epithelial loosening in the cornea bears certain obvious analogies to that of vesication in the skin. Some dissimilarities between the two processes have, however, already been revealed.

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VIII. THE EFFECT OF HISTAMINE AND RELATED SUBSTANCES ON THE COHESION OF THE CORNEAL EPITHELIUM*

HEINZ HERRMANN

In the preceding paper we described the use of a simple mechanical scraper with which it is possible to remove the epithelium of the excised cornea and to obtain at the same time a crude quantitative estimate of the adhesive forces. A decrease of the cohesion was observed when the corneas were bathed in weak solutions of higher aliphatic alcohols (butyl, amyl) and of some of their chemical derivatives, and a loosening was also observed on incubating for about 10 hours after injection into the cornea of some metabolic poisons such as iodoacetate or fluoride. These results made it seem desirable to find out whether the neurohormones and commonly used drugs produced similar effects. Too few compounds have been examined to allow for a systematic evaluation. However, we found that histamine differed in its effects from all the other drugs investigated so far, both qualitatively and quantitatively.[1] The results are published as a contribution to the pharmacology of histamine, since a continuation of the examination of other drugs is not planned at the present time.

RESULTS

The experiments were performed on beef corneas. The maintenance of the corneas, the supply of drugs, and the method for the determination of the adhesion have been described in the preceding paper. The adhesion of the epithelium was defined by the amount of epithelium removed as a function of the weight of the scraper. In fresh normal corneas very little epithelium (less than 30%) is removed at a pressure of 90 gms., and a weight of 200 gm. is required for complete removal. The greater the loss of cohesion, the smaller the weight

*The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

[1] With the exception of colchicine which causes loosening in equally low concentrations.

which is required for the removal of a certain amount of epithelium. When corneas are injected with saline, the cohesion decreases somewhat on incubation, corresponding to a range of weights of 60 gms. to 120 gms. for the minimum and maximum removal respectively. When 1 microgm. of histamine is injected into the cornea most of the epi-

TABLE I

The Effect of Histamine and Other Natural Bases on the Loosening of the Epithelium
Incubation: 20 hours at 29°C.

CONTROLS		REAGENT INJECTED	AMOUNT INJECTED PER CORNEA	PRESSURE IN GM.	% EPITHELIUM REMOVED
Pressure in grams	% Epithelium removed				
60	14, 20	Histamine	10 micrograms	40	90, 89
	32			40	92, 92
	13, 21		5 micrograms	40	86
			3 micrograms	40	85
95	45		1 micrograms	40	85, 66
					98, 61
			0.3 micrograms	60	42
				40	23
		Histidine	2 mg.	95	35
			1 mg.	60	25, 11
				95	45
		Arginine	1 mg.	60	12, 32
					9, 18
					8
		Asparagine	1 mg.	60	19, 17
60	28, 25	Tyramine	1 mg.	95	48, 28
			0.5 mg.	95	17
			0.1 mg.	95	25, 28
		Creatine	1 mg.	95	29
		Creatinine	1 mg.	95	23
95	8	Putrescine	0.5 mg.	95	18
60	11	Arginine +	1 mg.	40	91, 95
		Histamine	10 micrograms		67

thelium can be removed with a pressure of 40 gms., the lowest weight available on the scraper (Table I). The effect of histamine does not develop immediately but requires an incubation period of about 8 hours (Table II). None of the other substances tested which are chemically related to histamine, such as histidine, tyramine, and other natural bases, show any effect at all. Some of the other pharma-

cologically active compounds, adrenaline, ephedrine, physostigmine, however, cause a loosening, but the concentrations which are required are considerably higher than in the case of histamine. Whether the loosening action of high concentrations of physostigmine is due to its parasympathetic properties is doubtful since acetylcholine, as such or in the presence of low concentrations of physostigmine, has no loosening effect. A loss of adhesion after prolonged application of high doses of pontocaine and other local anaesthetics has been frequently observed clinically.

Histamine is remarkable not only because of the small quantities which suffice to cause a loosening of the epithelium, but also because it differs from the other effective substances in the mode of its action. If compounds which decrease the adhesion of the epithelium are in-

TABLE II

The Development of the Effect of Histamine during Incubation at 29°C.

10 Micrograms Histamine per cornea.

INCUBATION TIME IN HOURS	PRESSURE IN GRAMS	% EPITHELIUM REMOVED
1	60	10, 18, 8
4	60	5, 21, 19
10	60	55, 22, 74
20	40	82, 90

jected in a proper dose, the loss of the adhesion can develop to such an extent that it becomes possible to remove the epithelium as a coherent sheet. If this operation is carried out after injecion of fluoride or iodoacetate no epithelium remains on the surface of the stroma. After the injection of histamine a very thin film remains on the surface of the stroma which adheres firmly. This film consists of a single cell layer which remains on the stroma surface even after scraping with a pressure up to 60 gms. In histological preparations in which the epithelium was not removed after histamine injection, one can observe more frequently than in normal samples a fine discontinuity between the basal and the more superficial cells. Thus the actual separation in the case of histamine does not take place between stroma and epithelium but between the actively mitotic basal layer and the more inert superficial layer. It is interesting to notice that some

properties of the two surfaces of the basal layer seem to be controlled by different mechanisms.

The effect of histamine on the cohesion of the corneal tissue differs from other pharmacological actions of histamine by the long delay.

TABLE III

Effect of Various Drugs on Adhesion of the Epithelium

Incubation Time: 20 hours.

DRUG INJECTED AMOUNT PER CORNEA	INCUBATED AT 29°C.	
	Pressure in grams	% Epithelium removed
Pontocaine		
1 mg.	40	89, 84, 85
0.5 mg.	40	78, 71, 78
0.25 mg.	40	21, 23
0.1 mg.	60	15, 12
Ephedrine		
2 mg.	40	70, 87, 73
0.5 mg.	40	15, 31
	60	52, 55
0.1 mg.	60	15, 32
Adrenaline		
1 mg.	40	92, 87, 93
0.5 mg.	40	49
	60	45
0.1 mg.	60	34, 37
Physostigmine		
1 mg.	40	80, 72
0.5 mg.	60	31, 32, 28, 36
Acetylcholine		
0.5 mg.	60	27, 35
Acetylcholine 0.5 mg. + Physostigmine 0.25 mg.	60	18, 47, 32, 27

The possibility cannot be excluded that this lag period of up to ten hours may be due to very slow penetration. Alternatively one would have to assume that the action of histamine is an indirect one, e.g. by interference with the metabolism of the epithelial cells or by af-

fecting the cells at a certain physiologic state, e.g. during mitosis. The phenomenon which is reported here differs from many other pharmacological effects of histamine also in that it is not inhibited by arginine.

Possibilities for a more prolonged action of histamine are borne out by the investigations on histamine metabolism during pregnancy (1). It is of interest to speculate as to whether the mobilization of the placenta during normal pregnancy could be caused by mechanisms similar to the epithelial loosening in the cornea produced by histamine.

SUMMARY

1. Histamine in concentrations of 5–10 $\times$ 10^{-6} M. causes a loosening of the corneal epithelium. Chemically related natural bases such as histidine, tyramine, etc., have no such effect up to concentrations of 1 $\times$ 10^{-2} M.

2. Adrenalin, ephedrine, and physostigmine cause a loosening of the corneal epithelium in concentrations of the order of 10^{-3} M.

3. This histamine effect develops slowly after some hours of incubation. The loosening produced by histamine occurs at the boundary between the basal epithelial cell layer and the overlying epithelium, not between the basal cells and the underlying stroma as is the case with other agents which we have studied.

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IX. LOOSENING OF THE CORNEAL EPITHELIUM AFTER EXPOSURE TO MUSTARD*

HEINZ HERRMANN AND FAY H. HICKMAN

One of the prominent symptoms of mustard injury of the eye is a loosening of the corneal epithelium. This was found to occur not only in vivo, but also in the excised cornea incubated in a moist chamber after exposure to toxic agents. Consequently, it became possible to study the pathological process involved in the development of this symptom uncomplicated by inflammation or by reactions of the vascular and nervous systems and of other extracellular regulators. Some of the attributes of the cohesive mechanism have been analyzed by a method reported in the preceding papers. In the present experiments this method has been applied to the study of the effect of mustard on the adhesion of the corneal epithelium.

RESULTS

In the experiments to be reported here, beef eyes freshly obtained from the slaughter house were exposed to mustard vapor by resting the eye on the mouth of a cylindrical vessel 5 cm. deep, containing a thin layer of liquid mustard on the bottom. Analyses reported above (1) indicate that under these circumstances mustard enters the cornea at a rate of 0.6 micrograms per square centimeter per minute. The macroscopic appearance of the cornea changes surprisingly little after exposure of the tissue to mustard vapors for 10–20 minutes and subsequent incubation for 10–20 hours. After very severe exposures (approximately one hour) a moderate degree of corneal haze develops during incubation. With exposure of 20 minutes or more the corneas maintain their dome-like convexity better than do the controls, as if the rigidity of the stroma had increased. This phenomenon may be related to the loss of turgescence in the corneal stroma described by Cogan and Kinsey (2) after similar exposures.

The only macroscopically detectable alteration in the tissue after exposure for 15 minutes and subsequent incubation is a marked

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

loosening of the epithelium. The whole complex of inflammatory reactions which follow mustard exposure in tissues in vivo is here reduced to one single symptom.

1. *Exposure Time:* Using the vessels described in a preceding paragraph for the exposure, the minimum exposure time required for the production of the lesion is about 5 minutes. The full intensity

TABLE I

Adhesion of Epithelium after Exposure to Mustard Vapor for Various Periods

Separation: 2 strokes, 95 gm. pressure.

Incubation: 22 hours, 26–28°C.

CONTROL	EXPOSURE—MINUTES			
	5	10	20	60
	Per cent Epithelium Removed			
12	42	95	85	13
13	19	96	75	8
7	62	86	92	2
7	25	100		6
20	38	93		9
18	37	97		20
11	76	100		8
6	84	93		7
6	39	97		6
29				8
23				7
27				8
21				
19				
21				
19				
Average...16	47	95	84	9

of the loosening effect is observed after exposure for 10–20 minutes. However, if corneas were exposed for 60 minutes (Table I), no loosening could be observed in most experiments, and in no instance could loosening be observed when the exposure was extended to 120 minutes. An interpretation of this striking phenomenon will be attempted in the discussion later.

2. *Incubation Time:* The loosening of the epithelium develops

after a lag period. With an exposure of 10 minutes and subsequent incubation at 29–32°C., no reduction in the adhesion of the epithelium to the stroma can be detected during the first 5 hours, but the effect is fully developed after 10 hours incubation (Table II).

3. *Incubation Temperature:* When the incubation temperature was lowered, the loss of adhesion was suppressed. In the experiments in which the effect of a lowered incubation temperature was tested, the corneas were exposed to mustard for 10 minutes and then kept in a moist chamber for one half to one hour at room temperature. Following this, they were incubated at various temperatures for 20 hours and compared with controls (not exposed to mustard but incubated at the same temperature in a moist chamber). The exposed corneas

TABLE II

Adhesion of Epithelium after Varying Incubation Periods Following Exposures to Mustard Vapor

Exposure: 10 minutes.

Incubation: 26–28°C.

Separation: 2 strokes, 95 gms. pressure.

INCUBATION TIME IN HOURS AFTER EXPOSURE	PER CENT OF EPITHELIUM REMOVED					
	Control			Treated		
5	13	11	6	14	10	10
10	6	11	12	22	74	74
20	16	24		68	79	

incubated at 29°C. showed the usual decrease of the adhesion of their epithelium. Those incubated at 24°C. showed a much reduced effect. In those incubated at 11–18°C., no loss of epithelial adhesion could be observed (Table III).

As reported above, no loosening of the epithelium is observed during the first 5 hours of incubation at 29°C. The following experiment was performed in order to see how late in the symptom-free period cooling of the tissue could be instituted and still suppress the symptom. Beef corneas, after a standard exposure to mustard for 10 minutes were incubated at 29°C. for 4 to 5 hours. Thereafter they were divided into three lots. In one lot the adhesion was measured immediately and was found to be normal. A second lot was incubated at 10–18°C.

TABLE III

Per Cent Epithelium Removed after Incubation at Varying Temperatures

Exposure: 10 minutes.

Incubation Time: 20 hours.

Separation: 2 strokes, 95 gms. pressure.

CONTROL	EXPOSED TO MUSTARD VAPOR			
Incubation Temp. 29°C.	29°C.	22–24°C.	16–18°C.	11–14°C.
17	94	62	17	24
16	82	74	21	8
14	85	63	24	16
4	84	74	26	11
23	83	57		27
10	65	48		20
23	59	43		
11	66	39		
20	68	47		
17	76	43		
13	83	67		
16	88	24		
18	88	38		
8	69	39		
23	93	63		
17	86			
21	93			
20	85			
11	86			
14	82			
11	88			
77	87			
12	87			
11	87			
13	79			
	72			
	85			
	76			
	87			
	85			
Av. 15	86	52	22	18

for a further 15 hours. At the end of this period the epithelium was still normally adherent (Table IV). The third lot was incubated at 29°C. also for a further period of 15 hours. At the end of this period

this lot of corneas showed marked loosening of the epithelium. It is evident that cooling of the tissue can suppress the loss of adhesion even if cooling is applied near the end of the symptom-free period.

The effect of low temperature was tested in an additional series of experiments. Beef corneas were given a standard exposure to mustard vapor at room temperature, kept in a moist chamber at room temperature for one half to one hour, then kept for 6 to 8 hours in a cooled moist chamber (12–18°C.), and then incubated at 29°C. for 12 hours. Controls, with exposure to mustard vapor, were incubated at 29°C. also for 12 hours. We were thus able to test whether the period of cooling had any influence on the subsequent development of the lesion. Table V contains the results of all of the experiments performed of

TABLE IV

Incubation at Low Temperature Following Preliminary Incubation Period at 29°C.

Exposure: 10 minutes.

% EPITHELIUM REMOVED WITH 2 STROKES, 95 GRAMS PRESSURE		
Incubated for 4 hrs. at 29°C.	Incubated for 12 hrs. at 29°C.	Incubated for 4 hrs. at 29°C. and thereafter for 13 hrs. at 14–18°C.
18	67	10
11	87	15
26	81	17
6	85	21
	83	8
	81	

this type. While the samples that had been exposed to the preliminary cooling period gave rather widely scattered results, they show a statistically significant decrease in the degree of epithelial loosening, as may be seen by comparing the average percent epithelium removed with 95 grams weight on the scraper in the two series. The difference between these averages is 8 times the sum of their probable errors.

4. *Effect of Incubation under Anaerobiosis:* A second condition under which the loosening of the epithelium is suppressed after exposure to mustard was found to be incubation of the exposed corneas under nitrogen. For the anaerobic incubation the corneas were placed in a desiccator jar. The jar was evacuated for 15 minutes down to 50 mm. Hg, put into the incubator and then connected with a nitrogen

TABLE V

Per Cent Epithelium Removed after Incubation at 29°C., with and without Previous Incubation at 12–18°C.

Exposure: 10 minutes.

Separation: 2 strokes.

PRESSURE APPLIED, GRAMS	INCUBATION 12 HRS. AT 29°C. WITHOUT PRELIMINARY COOL PERIOD % EPITHELIUM REMOVED	PRESSURE APPLIED, GRAMS	PRELIMINARY INCUBATION		SUBSEQUENT INCUBATION 12 HRS. AT 29°C. % EPITHELIUM REMOVED
			Temp.	Hours	
		110	12	6	66
					62
					69
95	83	95	12	8	72
	87				46
	66		12	6	30
	76				40
	68				57
	64				32
	87				33
	81				36
	85		12	7	26
	83				52
	81		12	8	53
	87				48
	72				52
	85				41
	87				62
	76		14	8	73
	87				65
	85		18	8	71
					79
Av.....	80 ± 1.1				51 ± 2.4
80	87	80	18	8	69
	79				68
			16	8	71
60	90	60	16	8	71
	37				69
	12				64
	58				49
	63				30
	26				72
	63				28
	33				35
	49				51
	81				56
	56				37
	80				25
	58				66
	83				26
					83
					75
Av.....	55 ± 4				51 ± 3.3

tank. The gas was passed over copper wire at a temperature of about 500°C., and then bubbled through an alkaline pyrogallate solution in two separate containers, one inside, and the other outside the desiccator jar. A gentle flow of gas was maintained throughout the incubation period. In some experiments in which bicarbonate was used as an external bathing fluid for neutralization of the lactic acid which is formed anaerobically, nitrogen containing 5% CO_2 was used and the pyrogallate solution was omitted. In all of the corneas which were incubated for 10 hours under anaerobiosis after exposure to mustard for 10–20 minutes, the loosening was completely suppressed. The

TABLE VI

The Adhesion of the Corneal Epithelium under Aerobic and Anaerobic Conditions after Exposure to Mustard

Exposure: 15 minutes.
Incubation: 20 hours at 28°C.
Separation tested at a pressure of 90 gm.

CONDITIONS OF INCUBATION	NUMBER OF CORNEAS					
	Controls			Exposed to mustard		
	Epithelium					
	More than ½ off	About ½ off	Less than ½ off	More than ½ off	About ½ off	Less than ½ off
Aerobic	2	1	9	12	0	0
Anaerobic	0	0	13	0	0	13
10 hours anaerobic followed by 10 hours aerobic	1	2	13	6	4	8

same was observed with 1 ml. N/20 bicarbonate buffer as external bathing fluid which was used in order to prevent a too great drop of the pH due to the accumulation of lactic acid under anaerobic conditions.

As in the experiments with lower temperatures, we tried to obtain information as to whether the suppression of the loss of adhesion is limited to the period of anaerobic maintenance, or whether this effect is retained on readmission of oxygen. We incubated both exposed and control corneas for 10 hours anaerobically and subsequently for 10 hours aerobically, and compared these with control and exposed corneas which were incubated for 10 hours aerobically only. The complete experiment is shown in Table VI. All of the mustard

exposed corneas incubated aerobically showed loosening of the epithelium. In the series exposed to mustard and incubated first anaerobically and then aerobically, only about half showed loosening of the epithelium. This qualitative observation was supplemented by quantitative determinations of the removed epithelium which were carried out as described previously. The results of this experiment

TABLE VII

Adhesion of the Corneal Epithelium on Incubation under Aerobic and Anaerobic Conditions after Exposure to Mustard

Separation tested at a pressure of 90 gm. Removed and adherent epithelium from 3 corneas pooled in each sample.

Exposure: 15 minutes.

Incubation Temp. 29°C.

CONTROLS	% EPITHELIUM REMOVED
Incubated for 10 hours aerobically	13
Incubated for 20 hours anaerobically	13
	10
Incubated for 20 hours aerobically	7
	42
Incubated for 10 hours anaerobically followed by 10 hours aerobically	40
	42
EXPOSED	
Incubated for 10 hours aerobically	88
	89
Incubated for 10 hours anaerobically	9
	9
Incubated for 10 hours anaerobically followed by 10 hours aerobically	57
	59

agree with the qualitative findings (Table VII). None of the corneas which were kept for 20 hours anaerobically and all corneas which remained aerobic for this time showed the loss of adhesion. From these experiments it would seem that the effect of a preliminary incubation under anaerobiosis extends, in some cases, over a subsequent 10 hour period of aerobic maintenance. The results here are similar to those obtained on incubation at lower temperature. The

effect of anaerobic incubation on the epithelial loosening caused by iodoacetate, fluoride and histamine is shown in Table VIII.

TABLE VIII

Effect of Mustard and Other Agents upon the Adhesion of the Epithelium

Aerobic and Anaerobic Incubation

Separation with 90 gm. pressure.

SUBSTANCES TESTED	MODE OF APPLICATION	CONDITION OF INCUBATION	NUMBER OF CORNEAS		
			Epithelium		
			More than ½ off	About ½ off	Less than ½ off
Exposure to mustard vapor for 15′	No external bathing fluid	Aeroic	34	4	0
		Anaerobic	0	0	36
	Bathing fluid 1 ml. N/20 $NaHCO_3$	Aerobic	4	0	0
		Anaerobic	0	0	4
Fluoride 0.5 mg. per per cornea	External bathing fluid	Aerobic	23	0	0
		Anaerobic	0	0	22
	Injection into stroma	Aerobic	5	0	0
		Anaerobic	4	0	0
Iodoacetate 0.5 mg. per cornea	External bathing fluid	Aerobic	22	0	0
		Anaerobic	14	6	3
	Injected into stroma	Aerobic	5	0	0
		Anaerobic	4	0	0
Pontacaine 0.5 mg. per cornea	External bathing fluid	Aerobic	4	0	0
		Anaerobic	0	3	1
	Injected into stroma	Aerobic	4	0	0
		Anaerobic	4	0	0
Histamine 20 micrograms. per cornea	External bathing fluid	Aerobic	1	0	3
		Anaerobic	0	0	4
	Injected into stroma	Aerobic	4	0	0
		Anaerobic	0	0	4

DISCUSSION

Loss of cohesion between the corneal epithelium and stroma is a prominent feature of mustard injury. In vivo this symptom is

commonly associated with vascular and cellular inflammatory reactions. The experiments reported in this paper show that the loss of cohesion is not a consequence of inflammation and may be studied in vitro in the absence of any inflammatory reaction. Moreover, the loss of cohesion is not a manifestation of death of the epithelial cells. The experiments reported in one of the preceding papers (3) show that, even when the epithelium is loosened, the epithelial cells still maintain their capacity to respond by migrating in a completely normal fashion in the healing of small wounds. It is true that under ordinary experimental conditions, scattered cells in the basal layer show nuclear fragmentation and are evidently dying at the time that the loosening of the whole epithelial layer takes place. Nuclear fragmentation, however, has a larger temperature coefficient than epithelial loosening, and it is possible, by incubating at 25–30°C., to elicit the phenomenon of epithelial loosening without, or, at any rate, long before the appearance of nuclear fragmentation. Further evidence that the loss of cohesion is not a manifestation of tissue death is seen in the experiments which show that at lowered temperature or under anoxia the tissue is able to some degree to recover from the injury that otherwise leads to epithelial loosening.

Loss of cohesion between corneal epithelium and stroma appears to be an example of a rather widespread type of reaction to mustard injury. With adequate dosage the corneal endothelium sloughs off, and the loss of this cellular boundary leads to a severe oedema of the corneal stroma. Histologic study reveals that the capillary endothelium in the conjunctiva is similarly affected, and that after exposure to mustard many capillaries are converted into tubes devoid of endothelial lining. In many of these lesions petechial hemorrhages occur (4). In others the leakage of plasma through these denuded channels results in a close packing of the red cells and finally in a stoppage of circulation in the affected vessels. Similar changes are observable in the iris vessls after exposure to the more penetrating of the nitrogen mustards. To what extent this circulatory failure contributes to the development of tissue necrosis in severe lesions is still unclear, but it is certainly not without a significant effect on the whole pathologic process. Loss of cohesion between the epithelial cells has been shown by Danielli (5) to be an important factor in the production of vesication by mustard in the frog's skin, quite independent of the level of

extra-vascular tissue fluid pressure. To what extent all these pathologic processes are to be attributed to the same or similar mechanisms remains obscure. As noted in a preceding paper, the frog's skin and the beef eye behave differently in respect to loss of cohesion following exposure to certain metabolic poisons. Moreover, in the vesication in the skin produced by mustard, the loss of cohesion occurs between layers of epithelial cells, not primarily between epithelium and connective tissue as in the cornea.

In the development of all of the symptoms of mustard injury that have so far been studied, three phases are recognizable. There is first the primary chemical reaction of mustard with tissue components. In the cornea this is practically completed within a few minutes. There follows a symptom-free period during which neither physiological nor histopathological changes are recognizable. Poisoned nerves continue to conduct, muscles to contract, glands to secrete. The duration of the symptom-free period varies with the dose and with the symptom studied. In respect to epithelial loosening in the cornea the lag period is 6–12 hours.

If we speak of the mechanisms of mustard injury we assume that between the primary chemical reaction of mustard with tissue and the subsequent development of recognizable symptoms there exists a determinate series of events, but we are still almost completely in the dark as to that series of events. The present study has shown only that oxygen is required for the development of a particular symptom, but whether the symptom is due to the opening of some new oxidative channel in the cellular respiration, or to the inhibition of some normally compensating reductive process, remains obscure. Failure of the symptoms to develop after exposure to excessively large doses of mustard suggests that under these conditions the oxidative pathway, or some other system necessary for the development of this symptom, is inhibited.

The ability of fluoride and iodoacetate to produce epithelial loosening under anaerobic conditions indicates that these agents injure the normal mechanisms for the maintenance of tissue cohesion at some points other than, or in addition to, the injury produced by mustard. Since these agents are known to inhibit glycolysis, a first assumption would be that the glycolytic mechanism is involved in the maintenance of normal cohesion, but fluoride and iodoacetate, like mustard, damage

many unrelated intracellular mechanisms and, until more positive evidence is available, no specific conclusions can be drawn regarding the locus of their effect.

This analysis exhibits the pathological mechanisms of mustard injury as a process perhaps comparable in its complexity to that of normal physiological mechanisms, and suggests that the further study of this pathological mechanism may be useful in throwing light on the related physiological mechanisms. The obvious approach to such a study lies in the biochemical investigation of the tissue following mustard injury, and it is to this field that the succeeding papers in this series are devoted.

SUMMARY

1. Loosening of the corneal epithelium following exposure to mustard occurs both in vivo and in the isolated tissue incubated supravitally. It is independent of any inflammatory reaction.

2. The symptom develops after a period of incubation of 6–12 hours following exposure.

3. Lowering of the temperature or incubation in the absence of oxygen not only suppresses the symptom during the period of application of these conditions, but enables the tissue to recover partially from the injurious effect.

4. It is concluded that the symptom results either from some abnormal utilization of oxidative energy or from the inhibition of some normally balancing reductive process.

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X. EXPLORATORY STUDIES ON CORNEAL METABOLISM*

HEINZ HERRMANN AND FAY H. HICKMAN

The previously available knowledge on corneal metabolism is extremely limited, and has been reviewed in an earlier paper (1) from this laboratory. The present study was undertaken in order to establish some background on the basis of which metabolic effects of mustard injury could be sought for. These studies are in no sense complete. Several of the more interesting metabolic pathways discovered were not investigated further when it was found that they were unaffected by exposure of the tissue to mustard. Nevertheless, a general picture of the chief pathways of energy metabolism in the cornea has emerged.

For an investigation of the tissue metabolism the cornea offers the following advantages. It contains an endogenous store of reserve metabolites sufficient to maintain respiration and fermentation at the initial level in the excised tissue for at least one day. On the other hand, it is easily possible to supply to the cells of the excised tissue metabolites or inhibitors, either by irrigation, or by injection into the stroma. The latter procedure is of importance for the supply of relatively high concentrations of substances which are available in only small amounts. Because of its unique histological properties, metabolic rates can be measured without slicing or macerating the intact tissue. Furthermore, it is possible to separate the tissue components mechanically in order to use homogeneous samples of cells of special types, and to investigate possible metabolic interactions between tissue components. Experiments with corneas thus combine in many respects the advantages of metabolic studies on tissue slices and on whole organs, while at the same time furnishing almost uniquely simple opportunities for the study of intercellular metabolic processes.

METHODS

The experiments were performed on beef corneas. The preparation and supravital maintenance of the corneas have been described be-

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

fore (2). Determinations of extractable carbohydrates, lactic acid, and phosphates were carried out on trichloracetic acid extracts. Three corneas were pooled in each sample and were suspended in a total volume of 10 ml. of 5% trichloracetic acid. A satisfactory extraction requires vigorous and thorough grinding for 10 to 15 minutes.[1] Insoluble portions of the tissue were separated either by centrifugation, or by filtration through Whatmann Filter paper No. 42, (if phosphorus determinations are to be carried out).

Extractable carbohydrates were determined by Hoffmann's method (3); 0.2 to 0.5 ml. of the filtrates were used. The excess of acid was neutralized by addition of N/1 NaOH (yellow to thymolblue) and the volume was made up to 1 ml. with distilled water. 2 ml. of the ferricyanide reagent were added and the determination continued as described in the original procedure. This method is not very specific but it was satisfactory for our purpose, since in most experiments merely the rate of disappearance of an excess of injected glucose was measured.

Lactic acid was determined according to the method of Barker and Summerson (4).

Inorganic phosphorus was determined according to the method of Lohman and Jendrassik (5), adapted for use with photoelectric colorimeter (Klett Summerson Filter 660). The organic phosphate fractions were determined by differential hydrolysis, as described by Lohman (6). The hydrolysis was carried out with N/1 HCl in a boiling water bath. Three fractions were obtained, the first two after hydrolysis for 7 to 180 minutes respectively, and the third after digestion with concentrated perchloric acid for ½ hour. We found it to be convenient to hydrolyze the samples for each particular time individually. For this purpose aliquots of 0.5 ml. of the filtrate were pipetted into narrow 5 ml. serological tubes, and 0.5 ml. of 2N HCl was added. The tubes were closed with tightly fitting vaccine bottle stoppers and immersed in a boiling water bath. After the required time, the tubes were removed and cooled. The contents were transferred into Klett tubes, 1 ml. of the molybdate reagent and 0.2 ml. of the amino-naphthol-sulfonic acid reagent were added and the volume

[1] A mechanical device which can be used as a substitute for hand grinding in preparing corneal extracts is described in Appendix II.

made up to 5 ml. The samples which were digested with concentrated perchloric acid were also transferred to Klett tubes and the pH was adjusted to between 3 and 8 (yellow to thymolblue) by addition of about 0.8 ml. of 40% NaOH. Double amounts of reagents were added and the volume was finally made up to 10 ml. The color in all samples was developed in five minutes at 37°C.

Glycogen was estimated according to Sjögren's (7) modification of Pflüger's method. We found the determination to be practicable only when applied to the epithelium previously mechanically separated from the stroma. In the presence of stroma, extraction with alkali, the first step in the analytic procedure, yields large amounts of corneal mucoid in the extract, and attempts to precipitate glycogen by addition of alcohol to this mixture were unsuccessful. Epithelium from three corneas was digested in 0.5 ml. of 30% NaOH in boiling water bath for 30 minutes. The volume was brought to 1 ml. and 1.2 ml. of 95% alcohol were added. After 3 re-precipitations and hydrolysis in N/1 HCl for 3 hours, the amount of glycogen was determined as glucose with Hoffmann's method. In all colorimetric procedures standard samples were carried along for each set of determinations.

Oxygen uptake was measured in the Warburg apparatus with one cornea per flask bathed in 3 ml. M/20 phosphate buffer of pH 7.2–7.5. Anaerobic glycolysis was measured similarly using 3 ml. Ringer's bicarbonate solution for the bathing fluid in an atmosphere of 95% N_2 with 5% CO_2. The sodium hexosediphosphate used for the injection in some experiments was prepared from the commercially available calcium salt (Schwartz Lab) by precipitation of the calcium with an equivalent amount of sodium oxalate. All other reagents were the purest commercial samples. All solutions for injection were adjusted to pH 7.

In the case of the cornea, the use of the dry weight unit is misleading as the standard of reference for the calculation of metabolic rates. The inadequacy of such calculations for heterogenous tissues in general has been discussed by Berenblum et al. (8). These authors propose to relate the metabolic activity to the number of cells and to use the nucleic acid phosphorus as its chemical index. However, there does not seem to be any reason to assume that the nucleic acid content is an adequate index of the cell number since the nucleic acid content per

cell may vary greatly from tissue to tissue. In the cornea we had an opportunity to determine cell number and nucleic acid content in the stroma and in the epithelium, and to check in this way the assumed correlation. Microtome sections of the cornea cut vertically to the surface and of 8 micra in thickness were prepared. Cell counts were carried out in an area of $\frac{1}{2}$ mm. width. In the given area we obtained for the epithelium counts of 102, 112, 87, 114 cells, and for the stroma 56, 50, 56, 48.[2] The number of cells in the endothelium is about one-tenth that in the epithelium. As was pointed out in a previous paper (9), not all of the stroma cells are keratocytes. In the beef cornea 3–5% of the stroma cells are wandering cells.

The determinations of nucleic acid were carried out on the separated epithelium and stroma from several corneas according to the procedure described by Berenblum. We found about 40 micrograms of nucleic acid phosphorus in the epithelium and 10 micrograms in the stroma.[3] There is no correlation between these two sets of figures, and the nucleic acid content is apparently only a very crude index of the number of cells.

Since our metabolic studies have been carried out on the whole cornea and were aimed at a metabolic interpretation of the functional state of the histological units of the whole tissue, we considered the expression of the metabolic activity per cornea or per cell as the most adequate. Pooling 3 corneas for each determination and extending the number of the experiments yielded relatively stable mean values. In order to render the various systems of reference convertible, we have compiled all pertinent data, derived from our own determinations and from Krause's "Biochemistry of the Eye", in Table I.

EXPERIMENTAL RESULTS

(1) *Oxygen consumption.* Some data on the respiration of the cornea are available in the previous literature (1). For the purposes of the present study we have extended the previous results to include the O_2 uptake of the tissue at 32°C., since this was the temperature of incubation found most convenient for the study of the carbohydrate

[2] We are indebted to Dr. Buschke and Dr. Friedenwald for most of the histologic data.

[3] The value for the stroma is probably too high due to probable unavoidable contamination from the large amount of collagen in the tissue.

metabolism. At this temperature the oxygen uptake of the whole cornea was found to average 70 cu. mm. per hour. In corneas that had been denuded of their epithelium, the oxygen uptake was reduced to about 10%. If the endothelium is also removed less than 3% of the original oxygen consumption remains, and if this figure is corrected for the auto-oxidation found in boiled corneal stroma less than 2% of the respiratory uptake of the cornea remains to be attributed to the stroma, though the tissue contains approximately one-third of all the corneal cells. It must be remembered, moreover, that 3–5% of the stroma cells are wandering cells, and evidence has been presented in a previous paper (9) indicating that these wandering cells probably have

TABLE I

Reference Data on Beef Cornea

Whole Cornea

Greater Diameter	25– 30 mm.
Lesser Diameter	16– 21 mm.
Wet Weight	480–630 mg.
Dry Weight	90–120 mg. (19% of wet weight)

	EPITHELIUM	STROMA	ENDOTHELIUM
Wet weight	70–90 mg.	400–600 mg.	7–8 mg.
Dry weight	16–18 mg.	80–110 mg.	
Thickness	about 0.1 mm.	about 0.7 mm.	about 0.005 mm.
Number of cells	$4–10 \times 10^7$	$2–5 \times 10^7$	$1–2 \times 10^6$
Nucleic acid phosphorus	40 micrograms	less than 10 micrograms	

an aerobic metabolism since their pathological responses differ under aerobic and anaerobic incubation. If the oxygen consumption per wandering cell is equal to that per cell in the epithelium or endothelium, the total oxygen uptake of the stroma would be entirely accounted for. This leads to the conclusion that the keratocytes are probably completely devoid of respiratory oxygen uptake.

(2) *Glycogen.* The epithelium of 50 corneas was worked up according to Sjögren's method. After fivefold reprecipitation with alcohol, about 50 mg. of a white powder was obtained. A comparison of this substance with commercial glycogen (Schwartz' Lab) showed very similar rates of liberation of reducing groups during hydrolysis with HCl or with diastase, and an almost identical decrease in specific

rotation (Fig. 1). From the acid hydrolysates of both samples, phenylosazones were prepared which showed the characteristic crystal form of glucosazone and had a melting point of 203–204°C., which remained constant after mixing the two substances.

The corneal epithelium contains 1 to 1.5 mg. glycogen per cornea. In the isolated surviving cornea this carbohydrate reservoir is slowly depleted. The rate of depletion varies with temperature, being almost zero at 24°C. and about 25 micrograms per cornea per hour at 32°C.

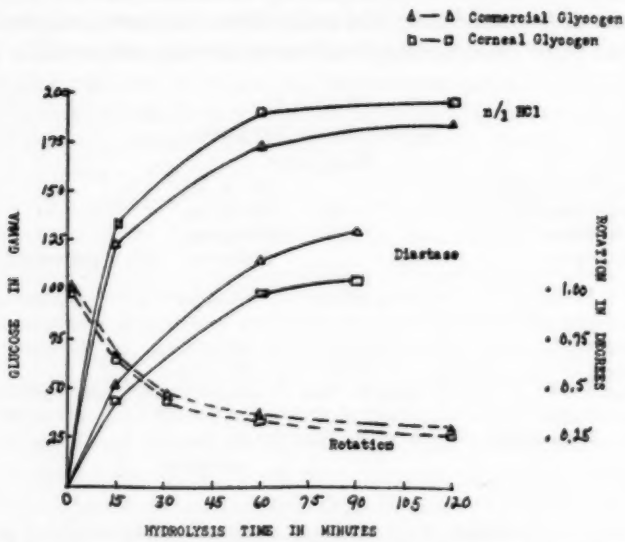

FIG. 1. COMPARISON OF HYDROLYSIS RATES OF COMMERCIAL AND CORNEAL GLYCOGEN.

(Table II). The rate of disappearance was found to be undisturbed by iodoacetate. After the injection of glucose the rate of disappearance of glycogen was diminished. No attempt was made to decide whether this effect was due to an inhibition of the breakdown of glycogen or to an accelerated resynthesis. However, even when as much as 10 mg. of glucose was injected into corneas no increase in the glycogen level was observed.

(3) *Glucose utilization and lactate content.* In vivo, the epithelium receives a continuous carbohydrate supply, in the form of glucose, from the tears (60 mg. % glucose) and through the stroma from the

TABLE II

Glycogen Content of Epithelium of Excised Corneas after Various Incubation Periods. Effects of Iodoacetic Acid and Glucose

Incubation at 32°C.

Micrograms Glycogen per Cornea

INJECTED SUBSTANCES MG. PER CORNEA	BEFORE INCUBATION	AFTER INCUBATION			
		10 hours		20 hours	
		Control	Injected	Control	Injected
No injection	908	848		600	
	1066	773		625	
	1100	833		575	
	1543				
	1088				
Iodoacetate, 0.5 mg.		866	966	608	516
		760	700	550	575
		785	991	566	508
		816	925	725	734
Glucose, 2 mg.	1175	640	1000	628	1050
		623	1025	391	1283
		833	1190		
		816	916		
		908	1250		
		941	1083		
Glucose, 10 mg.			1173		
			1106		

Average Glycogen content of fresh tissue, 1147 micrograms.
Average Glycogen content after incubation in presence of glucose, 1107 micrograms.
Glycogen consumption in the absence of injected glucose:

First 10 hours of incubation	34 micrograms per cornea per hour
Second 10 hours of incubation	22 micrograms per cornea per hour

capillaries in the limbus, and perhaps from the aqueous. We tried to imitate the natural conditions by irrigation of the cornea. The effects of two irrigation fluids, Ringer's solution and Ringer's plus glucose (0.1%) were compared.

The excised corneas were placed on glass slides (5 x 7.5 cm.) which were fastened to an inclined piece of sheet metal. Each cornea received the irrigation fluid from a separate 500 ml. Erlenmeyer flask through glass tubing and rubber connections, the number of drops being regulated by a screw clamp to a rate of about one drop every ten

TABLE III

Oxygen Uptake, Anaerobic Acid Production, and Lactic Acid Content of Beef Corneas After 23 Hours of Irrigation

Anaerobic Acid production measured as CO_2 output. 100 cu. mm. CO_2 is equivalent to approximately 400 micrograms of lactic acid.

The Lactic Acid content of "Controls" was measured after 23 hours irrigation without subsequent manometric measurements.

IRRIGATING FLUID	O_2 UPTAKE IN CU. MM. PER HOUR PER CORNEA	CO_2 OUTPUT IN CU. MM. PER HOUR PER CORNEA	LACTIC ACID CONTENT MICROGRAMS PER CORNEA	
Ringer	70		48	
	62		58	
		95	470	After 1 hr. Anaerobiosis
		99	560	
			62	Control
Ringer + 1% Glucose	62		295	
	68		630	
	61		510	
	58		220	
		121	1270	After 1 hr. Anaerobiosis
		105	860	
			335	Control
Ringer + Glucose + M/1000 Iodoacetate	42		63	
		22	13	After 1 hr. Anaerobiosis
		27	71	
			0	Control

seconds. Air pressure from a central regulator kept the dropping rate constant for several hours.

The corneas were maintained under irrigation for 20 hours at room temperature. Then the lactic acid content and the rate of respiration and of anaerobic glycolysis were determined. The two latter determinations were carried out in Warburg manometers with 2 half corneas per flask at a temperature of 38°C. (Table III). The rate of both O_2

uptake and anaerobic acid production after the irrigation period were found to be at the same level as in the fresh cornea. The addition of glucose has no apparent stimulating effect on the respiration, and even the anaerobic glycolysis is the same after the irrigation with Ringer's solution and with Ringer's plus glucose.

A great difference between the two irrigation fluids was observed in their effects on the lactate level of the irrigated corneas. A considerably higher lactate content was found in the corneas irrigated with the Ringer's solution which contained glucose. This effect of glucose is abolished if iodoacetate in a concentration of M/1000 is added to the irrigation fluid. In this case, the respiration was found to be inhibited by about 30% and the anaerobic glycolysis by 80%. Discrepancies of this type between the inhibitions by iodoacetate of oxygen consumption and of anaerobic acid production have been the subject of wide discussion (Barker, Shorr, and Malam (10)).

Extracts can be obtained from fresh excised corneas which reduce Hoffmann's Ferricyanide reagent. Some reduction takes place at room temperature and titrations with dichlorophenol indophenol indicate that about $\frac{1}{3}$ to $\frac{1}{2}$ of the total reduction values at high temperature are due to ascorbic acid. Pirie (11) has recently reported that almost all of the ascorbic acid in the cornea is in the epithelium, and our own experiments are in accord with her results. Calculated as glucose the total reducing values correspond to about 200–300 micrograms per cornea. Corrected for ascorbic acid this would correspond to 100 to 200 micrograms glucose per cornea. The total reduction value decreases during the incubation, and this change apparently concerns only the non-ascorbic acid fraction. However, no extensive experiments were carried out on the state of the endogenous reducing material. For a more quantitative study of glucose utilization in the excised cornea, we injected glucose into the stroma and observed its disappearance. We used amounts of glucose which were great compared with the quanitity of endogenous reducing material. In that way changes in the concentration of the latter during the incubation could be neglected.

We found that the injected glucose is rapidly utilized on incubation, about 100 micrograms disappearing per hour, per cornea (Table IV). Experiments with the separated stroma showed that considerable

TABLE IV

Glucose and Lactic Acid Content of Beef Cornea. Effect of Injected Glucose, Iodoacetate, and Fluoride

Incubation 10–12 hours, at 32°C.

	INJECTED PER CORNEA	GLUCOSE MICROGRAMS PER CORNEA		LACTIC ACID MICROGRAMS PER CORNEA	
		Before incubation	After incubation	Before incubation	After incubation
Whole Cornea	No injection	319	115		130
		352	116		135
		232	176		95
		165	117		90
	Glucose 2 mg.	1650	701		620
		1618	306		600
		1392	347		750
		1419	351		620
Denuded Stroma	Glucose 2 mg.	1660	1200	300	605
		1780	1120	350	565
			(20 hours incubation)		(20 hours incubation)
			840		550
			860		510
					505
					540
Whole Cornea	Iodoacetate 0.5 mg.		306		300
			264		255
	Iodoacetate 0.5 mg. Glucose 2 mg.		1285		285
			1171		255
	Glucose 0.5 mg.		160		203
			166		110
			200		223
			210		150
	Glucose 0.5 mg. Iodoacetate 0.5 mg.		543		143
			500		56
			580		40
			590		90
	Glucose 0.5 mg. Fluoride 1.0 mg.		323		370
			400		436
			320		400
			355		440
	Glucose 0.5 mg. Fluoride 1.0 mg. Iodoacetate 0.5 mg.		516		123
			586		110
					105

amounts of glucose are utilized in this tissue component. With only ½ of the cell number of the epithelium, the rate of utilization of glucose per cell seems to be about twice as high in the isolated stroma as in the epithelium.[4] The lactate content remains at the original level or increases slightly during incubation if as much as 2 mg. of glucose are injected per cornea. Smaller amounts of glucose (0.5 mg.) are not sufficient to maintain the lactate content at the initial level but retard its disappearance. Addition of iodoacetate inhibits the disappearance of glucose. 0.1 mg. of iodoacetate per cornea (about M/1000) still exhibits the full effect. The rate of disappearance of lactate in the presence of iodoacetate and glucose is the same as with iodoacetate alone and it is only slightly less rapid than in the untreated controls.

The inhibition of glucose disappearance by fluoride (about M/50) is less complete than that exhibited by iodoacetate, but the decrease of the lactate level is considerably retarded as compared with the effect of iodoacetate. This retardation is observed also in the absence of glucose. Iodoacetate applied in addition to fluoride increases the inhibition of the glucose disappearance and accelerates the disappearance of lactate. Thus it supersedes in both respects the action of fluoride. The retardation of the lactate disappearance both by iodoacetate and by fluoride will be considered in greater detail below.

(4) *Phosphate fractions.* The gradual depletion of the glycogen stores in the cornea during incubation, as well as the inhibiting effect of iodoacetate upon the rapid utilization of glucose, suggested the presence of active phosphorylating mechanisms, and led us to the examination of free and esterified phosphate in the cornea under various experimental conditions.

We determined 4 phosphate fractions. The phosphate which is determined directly without hydrolysis (IP) is equal to the sum of inorganic phosphate plus creatine phosphate, plus acetyl phosphate. The phosphate (ATP) liberated within the first 7 minutes of hydrolysis with HCl originates mainly from adenosine triphosphate, and the phosphate liberated during further hydrolysis up to 180 minutes (DP) is derived essentially from hexose diphosphate. The excess of phosphate found after digestion with perchloric acid is derived from such esters as phosphoglycerate (PG).

[4] More detailed studies in regard to glucose utilization are to be found in a succeeding paper (12).

The content of these fractions was determined in the whole cornea and in the epithelium and stroma separately, both in fresh corneas and after an incubation for 20 hours at 32°C. (Table V). One would have expected that the figures for the whole cornea should equal the

TABLE V

Extractable Phosphate Fractions

Micrograms Phosphorus per Cornea

	IP			ATP			DP			PG		
	Whole cornea	Epithelium	Stroma	Whole cornea	Epithelium	Stroma	Whole cornea	Epithelium	Stroma	Whole cornea	Epithelium	Stroma
Fresh tissue	35	32	22	16	8	3	28	28	2	31	24	18
	42	27	28	15	13	7	27	26	3	33	36	14
	24	28	21	26	18	2	18	14	7	30	39	18
	25	25	24	20	7	3	19	22	5	32	38	16
	26	24	23	19	11	4	29	27	3	42	33	23
	31	26	22	18	9	5	25	22	5	41	34	19
	34	27	21	23	8	3	33	29	7	35	24	25
	35	28	23	22	10	5	30	28	7	38	23	25
Average.....	31	27	23	20	11	4	26	25	5	35	31	20
After 20 hours,	18	16	18	17	14	3	19	14	5	34	30	17
Incubation at	22	19	19	14	12	4	24	16	7	32	31	28
32°C.	26	18	19	22	9	1	18	27	8	22	30	22
	25	16	24	11	13	2	34	23	12	34	23	25
	21	16	20	18	23	4	22	21	9	31	32	23
Average.....	22	17	20	16	14	3	23	20	8	31	29	23

IP = Phosphate determined directly i.e. inorganic phosphate + creatine phosphate + acetyl phosphate.

ATP = Phosphate determined after 7 minutes hydrolysis with N/1 HCl minus IP, i.e. chiefly adenosine triphosphate.

DP = Phosphate determined after 180 minutes hydrolysis with N/1 HCl minus (IP + ATP) i.e. chiefly hexose diphosphate.

PG = Phosphate determined after digestion with perchloric acid minus (IP + ATP+ DP) i.e. chiefly glycerophosphate.

sum of the values obtained for the separated epithelium and stroma. A good correspondence was observed with the fractions ATP and DP, of which a considerably higher concentration is found in the epithelium. These two fractions are bound to the cells, but even if calculated per

cell the concentration in the epithelium of ATP and DP are higher by 30% and 50% than in the cells of the stroma. In the IP and PG fractions, of the separated tissue components, we obtained values which were twice as high as the values for the whole cornea. Since the increase is not accounted for by a corresponding decrease in the other extractable fractions, a liberation of extractable phosphate from inextractable precursors has to be assumed. It is known that by slight damage various enzymatic reactions are started in the cell, e.g. aerobic glycolysis, and it is not impossible that the trauma of the separation activates enzymes which split IP and esters of the PG fraction from such inextractable precursors as nucleic acids, phospholipids, or phosphoproteins (13). This makes it impossible to give valid data on the distribution of PG and IP fractions between stroma and epithelium in the whole intact cornea. However, the epithelial cells are more exposed to injury in the course of the separation, the stroma cells being protected by the collagen sheets; consequently liberation of the extra P probably occurs in the epithelium. If this assumption is correct that most of the excess P is produced in the epithelium, the stroma would normally contain a much larger amount of these two fractions per cell than the epithelium. This seems probable since IP and PG are not necessarily bound to the cells and the excess could be due simply to the greater bulk of the stroma.

Values for fresh tissues change little on incubation. That this stationary state is not merely the result of a metabolic sluggishness of the cornea can be seen from the results in Table VI. They show that the levels of the various phosphate fractions in the cornea are sensitive to the influence of substrates and inhibitors. Marked alterations occur after injection of iodoacetate. The most prominent change takes place in the DP fraction, particularly when phosphate is supplied in addition to iodoacetate. Under these conditions the fraction increases by 40–50 micrograms per cornea. At the same time the IP fraction decreases by about 30 micrograms and the ATP fraction by 5–10 micrograms. Thus, practically the entire increase of the DP fraction is accounted for.

(5) *Lactate utilization.* In the preceding section, we showed that in vivo lactate is formed in the cornea by the continuous supply of glucose. For this reason an appreciable lactate content can be found in the freshly excised cornea which amounts to about 350 micrograms per

cornea; between one fourth and one fifth of this lactate content can be found in the epithelium, which forms only about one seventh of the total volume of the cornea. The concentration in the epithelium is, there-

TABLE VI

Phosphate Fractions in Whole Beef Corneas Effect of Iodoacetate

The figures represent the average values of 4–8 samples each.

INJECTED PER CORNEA		PHOSPHORUS IN MICROGRAMS PER CORNEA			
		IP	ATP	DP	PG
Sodium Phosphate 0.5 mg.	Examined before incubation	91			
No injection	Examined after 10 hours incubation at 32°C.	22	17	27	28
Iodoacetate 0.5 mg.		14	8	44	28
Iodoacetate 0.5 mg. + Phosphate 0.5 mg.		64	9	77	22

TABLE VII

Lactic Acid Content of Corneas after Varying Periods of Incubation at 32°C.

LACTIC ACID CONTENT IN MICROGRAMS PER CORNEA			
Incubation Time			
0 hours	4 hours	8 hours	11–12 hours
330	250	150	10
310	230	110	10
320		170	40
390		200	70
420		190	40
400		160	20
		170	40
			10
Av.....360	240	160	30

fore, somewhat higher than in the stroma. Without glucose supply, the lactate depot is gradually depleted at a rate of about 25 micrograms per hour per cornea, and after about 12 hours it has almost completely disappeared (Table VII). If, however, the epithelium is

first separated from the stroma and the two tissue components are incubated separately, then the lactate disappearance in the stroma, which contains about three fourths of the total amount present in the cornea, is practically eliminated and the lactate level in the separated

TABLE VIII

Lactic Acid Content Determined in Stroma and Epithelium Separately

Iodoacetate: 0.5 mg. per cornea.

		LACTIC ACID CONTENT IN MICROGRAMS PER CORNEA		
		Incubation Time in Hours		
		0	8	22
Stroma and Epithelium separated prior to incubation	Epithelium		250	266
			300	327
	Epithelium + Iodoacetate		90	
			30	
	Stroma		318	209
			259	126
			375	
			275	
	Stroma + Iodoacetate		260	
			300	
Stroma and Epithelium separated after incubation	Epithelium	84	36	27
		68	59	18
		80	45	
	Stroma	324	153	0
		316	42	5
		301	210	
			243	

epithelium seems to increase (Table VIII). However, if iodoacetate is added, the normal lactate disappearance is found in the separated epithelium while no effect of iodoacetate can be observed on the inhibited lactate disappearance in the stroma. This could mean that in the case of the epithelium, the increase is due to the aerobic glycolysis which ensues after cell injury. Such a process is unlikely in the case

of the stroma because here the cells are protected from injury by thick covers of collagen.

The dependence of the lactate level in the whole cornea upon glucose and iodoacetate is demonstrated in a greater number of experiments

TABLE IX

Effect of Varying Iodoacetate Concentrations upon the Lactic Acid Content of Corneas

Incubation Time: 10 hours.

IODOACETATE INJECTED PER CORNEA	LACTIC ACID CONTENT IN MICROGRAMS PER CORNEA
0	100, 105
0.25 mg.	120, 150
0.5 mg.	190
1.0 mg.	170

TABLE X

Lactic Acid Content in Corneas after the Injection of Glucose, Iodoacetate, and Glucose + Iodoacetate

Incubation: 12 hours at 32°C.

MICROGRAMS LACTIC ACID PER CORNEA							
Control		Iodoacetate .5 mg. per cornea		Glucose mg. injected per cornea			Glucose (2 mg. per cornea) + Iodoacetate (.5 mg. per cornea)
				6.0	2.0	1.0	0.6	
50	130	180	160	470	420	330	180	170
90	40	70	250	450	210	440	220	220
80	110	150	240	530	390	310	170	200
30	110	150	80		500	430		190
20	170	130	150		590	340		180
90	40	90			37	330		240
80	80	80			42			200
100	40	110			180			250
60	130	170						
60		180						
Av....	80	146		480	385	363	180	206

listed in Tables VIII to X. In the whole cornea iodoacetate itself inhibits the lactate disappearance only slightly (Table IX), but it restores the lactate disappearance after its suppression by glucose (Table X). Glucosamine shows a similar effect to that of glucose

(Table XI). This seems to indicate that glucosamine is converted into lactate in the cornea or is metabolized through a mechanism which interferes with the lactate utilization. Here, too, iodoacetate restores the original rate of lactate disappearance.

The similarity of the effect of glucose and glucosamine upon lactic acid disappearance was surprising. Lutwak-Mann (14) recently studied the oxidation of this amino sugar by various tissue prepara-

TABLE XI

Lactic Acid Content in Corneas after the Injection of Glucosamine, Iodoacetate, and Glucosamine + Iodoacetate

Incubation: 12 hours at 32°C.

LACTIC ACID MICROGRAMS PER CORNEA					
Control	Iodoacetate .5 mg. per cornea	Glucosamine mg. injected per cornea			Glucosamine (2 mg. per cornea) + Iodoacetate (0.5 mg. per cornea)
		6.0	2.0	0.6	
50	180	160	260	240	300
90	70	230	330	190	100
80	150	290	250	260	190
30	130	130	280	200	240
20	240	270	370	210	150
80	80	290	300	350	190
90		170	350	220	300
60		180	420	220	270
130		170			110
40		300			230
40					200
80					
40					
Av...64	150	230	320	236	207

tions. Under the experimental conditions used by this author, neither lactate nor pyruvate could be found among the breakdown products. The elucidation of the metabolism of glucosamine in the cornea is desirable, since this amino sugar is a constituent of the mucopolysaccharides which are of importance for the maintenance of the transparency of the cornea. Among a larger series of potential lactate precursors, other than glucose and glucosamine, only hexosediphos-

TABLE XII

Lactic Acid Content of the Cornea after Injection of Various Metabolites

Incubation: 10 hours at 32°C.

INJECTED SUBSTANCE PER CORNEA	MICROGRAMS LACTIC ACID PER CORNEA	
	Control	Injected
Hexose diphosphate, 2 mg.	76 60 73	233 220 243
Ascorbic Acid, 2 mg.	73 60	50 60
dl Alanine, 2 mg.	47 90	138 170
Glycine, 2 mg.	90	75
Glutamic Acid, 2 mg.	90	96 90
Phosphate, 0.5 mg.	93 121	100 108
Succinate, 0.5 mg.	80 80	60 70
Fumarate, 0.5 mg.	130	110 140 140
Malonate, 0.5 mg.		110 130

TABLE XIII

Effect of Fluoride on Lactic Acid Content of Cornea

Incubation: 10 hours at 32°C.

FLUORIDE INJECTED PER CORNEA	MICROGRAMS LACTIC ACID PER CORNEA
None	85, 57, 99, 95 60, 40, 123, 130
0.01 mg. 0.03 mg. 0.1 mg. 1.0 mg.	260 320, 258, 242, 145 432 326, 263, 316, 378, 425
Iodoacetate 0.5 mg. Iodoacetate + 1 mg. Fluoride	100, 73, 100, 131, 83 176, 150, 156, 111, 178

phate had a qualitatively similar effect, although quantitatively less intense, upon the lactate disappearance (Table XII).

Other conditions, under which the inhibition of lactate disappearance is removed by iodoacetate, are injection of fluoride (Table XIII) and lower temperature (Table XIV). An explanation of this apparently paradoxical effect will be attempted in the following discussion.

TABLE XIV

Lactic Acid Content of Corneas after Incubation for 12 Hours at Room Temperature with and without Injection of Iodoacetate

Injected: 0.5 mg. Iodoacetate per cornea.

MICROGRAMS LACTIC ACID PER CORNEA	
Controls	Iodoactate
240	146
223	170
256	156
245	141
Av.....241	153

DISCUSSION

In the experimental part of this paper, several compounds have been demonstrated in the corneal epithelium, which are expected to be present in a tissue with an active glycogenolysis or glycolysis. The glycogen content of the epithelium and of muscle are nearly equal (1%) and the phosphate fractions are found to be of the same order of magnitude as in the intestinal mucosa (15). A compilation of the adenosine polyphosphate level of various organs (16) shows that the epithelium of the cornea contains more of this fraction than liver, kidney, and brain and approximately as much as muscle. The lactic acid content is about 5–7 times higher in the epithelium (200 mg. %) than it is in the blood under conditions of rest and it also exceeds the content of the blood after severe exercise (17).

Most of these figures represent steady states, the level of which depends upon the rate of formation and consumption of the substance under observation. A shift of these levels will become evident if either formation or consumption are interfered with; simultaneous inhibition

or acceleration of both will lead to a change in the rate of turnover of the respective compound but its level will not necessarily deviate from the initial value.

The glycogen content of the epithelium decreases at a rate of about 25 micrograms per hour. In 20 hours about 50% of the initial content has disappeared. Approximately the same rate of disappearance has been found for the liver of rat and dog, whereas in the liver of the pigeon as much as 75% can be recovered after incubation for 72 hours (18).

In tissues of animals, glycogen is supposed to be converted by phosphorolysis (19) into hexose-1-phosphate, which in turn is transformed into hexose-6-phosphate and then to hexosediphosphate. The phosphorolysis is not inhibited by iodoacetate (20), and in the presence of this substance the glycogen of the corneal epithelium disappears at the same rate as in controls. However, if iodoacetate is added, an increase of hexosediphosphate (DP) fraction is observed and the increase is greater when inorganic phosphate is added. This effect could be explained by the well known inhibition of triosephosphate dehydrogenase by iodoacetate and the consequent displacement to the left of the enzymatic equilibrium:

$$\text{Hexosediphosphate} \rightleftharpoons \text{Triosephosphate} \rightleftharpoons \text{Lactate}$$

The undiminished rate of disappearance of glycogen and the simultaneous increase of slowly hydrolizable phosphate esters after addition of iodoacetate seemed to corroborate the assumption of a phosphorolysis. We failed, however, to demonstrate an inhibition of the glycogen disappearance by phloridzin, which is supposed to inhibit the phosphorolysis (21). This result cannot be regarded as a serious objection against phosphorolysis, since it has not been demonstrated that the corneal epithelium is sufficiently permeable for this inhibitor.

Glucose does not disappear in the presence of iodoacetate which would indicate that under these circumstances phosphorylations which require energy do not occur to any large extent. It is therefore, possible that the entire excess of phosphate in the DP fraction is hexosemonophosphate and derived from the phosphorolysis of glycogen and not diphosphate, the esterification of which would require, at least indirectly, oxidative energy. With this assumption, the appearance of an excess of 20 micrograms phosphorus in the DP fraction should

correspond to about 120 micrograms of hexose. We found, however, a disappearance of 200–250 micrograms of glycogen during the same time interval. This difference could possibly be explained by the assumption that only a part of the hexosemonophosphate is split up under our conditions of hydrolysis, which is possible according to Lohman's data. Alternative possibilities for the removal of glycogen are its enzymatic hydrolysis or a direct oxidation of hexosemonophosphate. Neither one of these hypothetical pathways is sufficiently well known to make a discussion here profitable. The investigations on oxidation of hexosemonophosphate have recently been reviewed by Krebs (22).

Glucose is rapidly metabolized in the supravital cornea. About 1000 micrograms disappear in the course of 12 hours. This disappearance is practically completely inhibited after addition of iodoacetate. By the inhibition of triosephosphate dehydrogenase and by the consequent suppression of the oxidation of triosephosphate and its breakdown products, iodoacetate prevents phosphorylations which require the supply of oxidative energy. The phosphorylation of glucose is one of such reactions and it is, therefore, understandable that in the presence of iodoacetate no hexosephosphate accumulates in excess of the amount which is found in the absence of glucose and that the disappearance of glucose is inhibited. Since no oxidative energy is required for the phosphorylation of glycogen, the formation of hexosephosphate from this precursor remains undisturbed by the addition of iodoacetate. The coupling of oxido-reductions with phosphorylation has been discussed in detail in two extensive reviews (16, 23).

Fluoride inhibits the disappearance of glucose to a lesser degree than does iodoacetate. In agreement with the results of other authors, it seems likely that enolase in our tissue preparations is only partially inhibited by fluoride (24).

The influence of glucose on the lactic acid level is demonstrated by the high lactate content of the fresh cornea immediately after interruption of the natural glucose supply; by the maintenance of this initial value by irrigation or injection of glucose; and by the restoration of the considerable rate of disappearance of lactate if iodoacetate is present in addition to glucose.

In the conventional scheme for the carbohydrate breakdown, lactate is formed by the reduction of pyruvate. According to the scheme proposed by Ball (25), pyruvate can compete for the hydrogen of reduced diphosphopyridine-nucleotide (DPN) with those "hydrogen carrying" systems which eventually reduce molecular oxygen. If the rate of reduction of DPN exceeds the capacity of the intermediate systems which react with oxygen, pyruvate substitutes as oxidant and is reduced, with formation of lactate. Lactate, on the other hand, is oxidized to pyruvate if sufficient oxygen is available or if the rate of reduction of DPN by other competing systems is diminished.

In vivo, glucose is constantly supplied to the epithelium, the diphosphopyridine-nucleotide (DPN) is presumably reduced at a high rate, and the reaction:

$$\text{Pyruvate} + H_2 \rightleftharpoons \text{Lactate}$$

moves to the right. The same will be the case in vitro, if glucose is supplied by injection or by irrigation. If iodoacetate is added, the oxidation of triosephosphate and the simultaneous reduction of DPN is inhibited, the consumption of glucose is prevented, no pyruvate is formed from glucose, and the oxygen supply will be ample to oxidize small amounts of DPN which are being reduced by other sources. The reaction:

$$\text{Pyruvate} + H_2 \rightleftharpoons \text{Lactate}$$

will then be displaced to the left.

In this discussion, three oxidative pathways are discernible which can participate in the regulation of the lactic acid level. The first is the oxidation of DPN, which is reduced by triosephosphate; the second is the system which oxidizes pyruvate; the third is the oxidative mechanism for the oxidation of DPN, which is reduced in the transformation of lactate to pyruvate. Within the structural setup of the intact cell, the first and last pathway might or might not be identical.

The total reserves of carbohydrates and carbohydrate derivatives which can be oxidized by the excised cornea consist of glycogen, glucose, and lactate. In one hour one cornea has an oxygen uptake of about 70 cu. mm. (32°C.) or about 3.1×10^{-3} mM O_2. In the same time interval the cornea consumes about 100 micrograms of glucose. The figures match exactly since for the complete oxidation of 100 micrograms of carbohydrate 73 cu. mm. O_2 are required. How-

ever, the small amount of endogenous glucose in the cornea is exhausted in 1–2 hours, and thereafter the carbohydrate consumption amounts to 25 micrograms glycogen plus 25 micrograms lactate. The remaining lactate disappears in about 12–20 hours. A deficit of oxidizable carbohydrates should ensue as soon as glucose has disappeared, this deficit becoming more marked after exhaustion of the lactate storage, since the rate of oxygen uptake is maintained at the initial level for at least 24 hours. This would indicate that as long as glucose is supplied to the cornea carbohydrate breakdown suffices for the maintenance of the oxidations, and in vivo the supply of glucose from the tears and through the limbal capillaries should prevent a deficit of oxidizable carbohydrate derivatives. Although the disappearance of lactate and glycogen is suppressed in the presence of an excess of glucose, the rate of glucose utilization is sufficiently fast to provide the required amount of oxidizable substrate. Once the glucose is exhausted, as is the case in the excised cornea after one or two hours, one half of the oxidizable substrate and, after disappearance of lactate, three fourths of the oxidizable substrate have to be derived from other sources than carbohydrates, presumably from fats or proteins.

The fact that the rate of oxygen uptake is maintained in vitro, possibly at the expense of material other than carbohydrate, could explain the fact that inhibitors of glycolysis, like iodoacetate, do not completely suppress the rate of respiration in this tissue (26).

The above considerations possibly provide a clue for the explanation of the effect of iodoacetate on the lactate disappearance. We observed in our experiments that the normal rate of lactate utilization in the excised cornea is retarded to a very slight extent by iodoacetate. In the presence of glucose or fluoride, or on incubation at a lower temperature, the lactate disappearance is practically completely inhibited, and is restored under these conditions by iodoacetate to almost the normal rate.

From the calculations in the previous paragraphs we have arrived at the conclusion that in the excised cornea glycogen and lactate together do not provide sufficient substrate for the saturation of the oxidative mechanisms. Therefore, the lactate oxidation can proceed at a maximum rate. However, when glucose is present and the reduction of oxidizable coenzyme by triosephosphate dehydrogenase

is accelerated, or when the oxidations are inhibited to a greater extent than the glycolytic mechanism, the triosephosphate dehydrogenase will compete for oxidized coenzyme with the lactic acid dehydrogenase and the lactate disappearance will be inhibited. In both instances iodoacetate should restore lactate consumption to about the normal rate since the competitive reduction of oxidized coenzyme by triosephosphate dehydrogenase is eliminated.[5]

Another aspect of the oxidation of lactate in the excised cornea should be discussed here. Our experiments show that lactate production from carbohydrate precursors takes place at a comparable rate in both the epithelium and in the stroma. The systems for enzymatic oxidation, however, are restricted to the epithelium, while the metabolism of the separated stroma is essentially anaerobic. In the fresh cornea three fourths of the lactate depot in the cornea are found in the stroma and the lactate content in the denuded stroma remains practically unchanged for as long as 20 hours. In the whole cornea, with the epithelium covering the stroma surface, the lactate depot in the stroma is depleted at a rate of 25 micrograms per hour, as mentioned above. Lactate from the stroma is, therefore, utilized by the epithelium and oxidized there through the cytochrome oxidase system, the presence of which in this tissue component has been demonstrated in a previous paper (1). The dependence of the lactate level in tissues upon oxygen supply is known as the "Pasteur effect". In our case the lactate level is regulated by the metabolic interdependence of the two histological components of a tissue and one could speak, therefore, of a "histological Pasteur effect". Such metabolic interaction might be of significance in many cases where stroma and epithelium form the main histological elements in an organic unit. The unequal distribution of oxidative enzymes in some tissues supports this suggestion. Lactate transfer and oxidation need not be the only metabolic interaction between an oxidatively very active tissue and an adjacent tissue with anaerobic metabolism. Studies previously published from this

[5] We believe that it is permissible to omit in this discussion the role of the oxidation of pyruvate itself because this substance never accumulates in the cornea. When injected in large excess it is removed at an extremely fast rate of about 400 micrograms per hour and per cornea, by a non-oxidative mechanism which will be described in greater detail in one of the following papers (27).

laboratory indicate that in the ciliary body (28) and in the choroid plexus (29) an actual oxidative interchange takes place between epithelium and stroma through the mediation of a system of oxidation-reduction carriers. The more highly integrated economy resulting from such specific and extensive interactions may be imagined as one of the characteristics of tissue organization.

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XI. THE EFFECT OF MUSTARD ON SOME METABOLIC PROCESSES IN THE CORNEA*

HEINZ HERRMANN AND FAY H. HICKMAN

In the preceding paper we have reported our preliminary studies on the metabolism of the cornea, studies which enabled us to establish a crude balance between the rate of reaction in the glycolytic pathway and the rate of oxidation of the various oxidizable carbohydrate breakdown products. We were, consequently, in a position to test whether any of the metabolic pathways so far disclosed were affected by mustard. Previous experiments (1) on the loosening of the corneal epithelium by mustard had indicated that there might be a connection between metabolic oxidations and the development of the lesion. We were particularly interested, therefore, in finding out whether doses of mustard which produce this lesion also produce a recognizable disturbance in the metabolism, and whether or not such disturbances preceded the development of the lesion.

TECHNIQUE AND RESULTS

The exposure of the tissue to mustard and the supravital maintenance of the cornea have been described before (1). The biochemical techniques are given in the preceding paper (2). The experiments were performed on beef corneas.

(1) *Oxygen Uptake.* Our results clearly indicate an inhibition by mustard of the oxygen uptake of the cornea (Table I). The inhibition first appears after 3–4 hours of incubation. Exposure to mustard vapor[1] for 5 to 20 minutes and subsequent incubation at 32°C for 5 to 20 hours prior to measurement in the Warburg apparatus revealed a decrease in the oxygen uptake of about 25%, and this decrease remained fairly constant over a relatively wide range of experimental conditions. A more complete inhibition of oxygen uptake (50% or

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

[1] Under the conditions of our experiments the mustard uptake was estimated as 0.6 micrograms per cm^2 per minute.

more) was found after exposure to mustard for 60 minutes. It should be noted that control corneas incubated for as long as 19 hours showed some increase of O_2 uptake when compared with fresh tissue or with samples incubated for shorter periods. The onset of this enhanced O_2 uptake appears to coincide with the depletion of the lactate reserves noted in the previous paper and is, no doubt, associated with the oxidation of non-carbohydrate components of the tissue.

(2) *Glycogen.* There is a slight transitory increase in the rate of glycogen consumption during the first few hours after exposure to mustard. Thereafter no significant difference in the rate of glycogen

TABLE I

Effect of Mustard on the Oxygen Uptake, Anaerobic Acid Production and Lactic Acid Content of Beef Corneas

Incubation: 19–22 hours at 32–33°C.
The figures given each represent the average of 3–15 samples.

EXPOSURE TO MUSTARD, MINUTES	O_2 UPTAKE PER CORNEA CU.MM. PER HOUR	% INHIBITION O_2 UPTAKE	ANAEROBIC CO_2 OUTPUT CU.MM. PER CORNEA PER HOUR	LACTIC ACID CONTENT MICROGMS. PER CORNEA
0	84		67	30
5	54	27	77	137
0	73		86	69
20	45	25	87	445
0	83		76	85
120	40	52	52	455

consumption was noted between controls and mustard treated corneas. In the presence of excess glucose, neither controls nor mustard treated tissues showed a reduction in their glycogen content (Table II).

(3) *Anaerobic Glycolysis.* Anaerobic acid production was tested on tissues exposed to mustard, incubated in the presence of oxygen for various periods, and then suspended in the Warburg apparatus in Ringer's bicarbonate solution in an atmosphere of 95% N_2 and 5% CO_2. The preliminary aerobic incubation was used in order to avoid the possibility that the development of the mustard injury might be inhibited by anaerobiosis, as we had found to be the case in respect to the loosening of the corneal epithelium. No difference was found

between tissues exposed to moderate doses of mustard and controls (Table I). Confirmatory evidence that the earlier steps in the glycolytic mechanism are not abnormal after exposure to mustard is found in the fact that iodoacetate, used in addition to mustard, gives the same inhibition of glucose consumption and the same increase in the hexose phosphate fraction (Table VI) as it does in the absence of mustard.

(4) *Lactate Utilization.* By far the most pronounced metabolic effect which we observed in the cornea on exposure to mustard is an inhibition in the lactate disappearance (Table III). Since there was no parallel increase in the rate of consumption of lactate precursors

TABLE II

Glycogen Content of Corneal Epithelium after Exposure to Mustard

The figures given each represent the average of 3–15 samples.
Incubation at 32°C.

	CONTROL	EXPOSED
Exposure 10 minutes		
Incubation time		
10 hours	926	799
20 hours	589	519
Exposure 15 minutes		
Incubation 20 hours	783	722
+ 2 mg. glucose	1052	1106

(glucose and glycogen), we concluded that the failure of the lactate reservoir to become depleted on incubation was due to an inhibition in the utilization of lactate, not to an enhanced rate of production. If iodoacetate is applied in addition to mustard, the lactate utilization is reestablished, though at somewhat slower rate than in the absence of mustard. The interpretation of this effect will be discussed in a subsequent paper (3).

Exposure of the cornea to mustard vapor for 3 minutes results in a slight inhibition of lactate disappearance (Table IV). After exposure of 5 minutes the rate of lactate disappearance was reduced by somewhat more than 50 %. Exposures of 15–20 minutes brought the disappearance of lactate to a complete standstill. Since in normal

controls there is a measurable loss of lactate on incubation for 2 hours, our failure to detect any loss of lactate following exposures of these

TABLE III

Lactic Acid Content of Beef Corneas. Effect of Mustard, Iodoacetate, and Mustard + Iodoacetate

Exposure to mustard vapor: 5–10 minutes.
Incubation: 12 hours at 32°C.

MICROGRAMS LACTIC ACID PER CORNEA			
Control	Iodoacetate .5 mg. per cornea	Mustard	Mustard plus Iodoacetate
80		360	
90	150	360	
60		240	
70		330	160
160		250	350
80	130	300	210
100	90	360	180
60	80	310	220
60	110	270	170
110	160	410	280
170	150	440	150
	250	300	220
		330	260
Av.....93	140	328	220

TABLE IV

Effect of Mustard on Lactic Acid Content of Beef Corneas. Variation in Exposure Time

Incubation: 10 hours at 32°C.

MICROGRAMS LACTIC ACID PER CORNEA			
EXPOSURE IN MINUTES	WHOLE CORNEA	EPITHELIUM	STROMA
0	170	22	36
3	185	15	85
5	240	45	260
10	315		

magnitudes indicates that the lag period in the onset of inhibition of lactate utilization following exposure to mustard is less than two hours.

TABLE V

Effect of Mustard on Extractable Phosphate Fractions

Incubation time: 10–12 hours at 32°C.

MICROGRAMS PHOSPHORUS PER CORNEA

IP			ATP			DP			PG		
Control	5′ Treatment	10–20′ Treatment	Control	5′ Treatment	10–20′ Treatment	Control	5′ Treatment	10–20′ Treatment	Control	5′ Treatment	10–20′ Treatment
22		23	24		27	25		14	34		38
20		25	14		23	22		13	33		43
26		27	13		17	24		35	31		41
30		28	15		23	34		31	32		37
21		33	17		26	37		22	38		44
31		32	18		27	25		18			42
19		25	15		27	22		14			
19		24	19		28	20		13	32		47
24		33	28		23	14		11	38		40
25		31	15		32	25		4	42		39
25		28	21		28	17		11	34		34
26		30	14			17	15		19		25
22	22	28	12	29	22	23	15	26	36		33
17	25	27	15	23	27	25	24	6	29		40
19	25	25	15	17	28	22	21	7	28		32
19	26	25	13	20	31	23	17	7	28		25
23	28		14	28		28	24		22	38	35
25	28		23	18		20	23		30	35	37
24	23	24	26	19	23	20	20	23	34	33	42
	23			24			18			36	
23	22	21	19	22	25	23	20	22		34	40
	23			21			22			36	
25		27	17		13	24		34			
23		36	18		18	24		18	27		45
24		30	12		28	21		22	35		41
28		31	16		17	21		34			
22		29	18		22	23		26			
25		28	20		30	18		17			
22		22	25		30	22		16			
27		31	26		32	17		22			
21		24	22		26	20		25			
21		25	18		24	23		22			
22		26	19		28	20		22			
25		30	17		25	27		21			
Av. 23	24	28	18	22	25	23	19	19	32	35	40

Total Extractable Phosphate	Control	5′ Treatment	10–20′ Treatment
Micrograms Phosphorus per Cornea	96	100	112

IP = Phosphate determined directly i.e. inorganic phosphate + creatine phosphate + acetyl phosphate.

ATP = Phosphate determined after 7 minutes hydrolysis with N/1 HCl minus IP, i.e. chiefly adenosine triphosphate.

DP = Phosphate determined after 180 minutes hydrolysis with N/1 HCl minus (IP + ATP), i.e. chiefly hexose diphosphate.

PG = Phosphate determined after digestion with perchloric acid minus (IP + ATP + DP), i.e. chiefly glycerophosphate.

This effect is, therefore, among the earliest of the reactions to mustard so far recognized.

These results are to be compared with those of our experiments previously reported on the loosening of the corneal epithelium following

TABLE VI

Extractable Phosphate Fractions. Effect of Mustard, Iodoacetate, and Inorganic Phosphate

Exposure to mustard vapor: 10 minutes.
Incubation: 10–12 hours at 32°C.
Iodoacetate injected 0.5 mg. per cornea.
Sodium phosphate injected 0.1 mg. per cornea.

	MICROGRAMS PHOSPHORUS PER CORNEA					
Phosphate Fractions	Control	Iodoacetate	Phosphate	Iodoacetate + Phosphate	Iodoacetate + Mustard	Iodoacetate + Mustard + Phosphate
IP	22	13			17	
	25	15			11	
	20	12				
	26	13				
ATP	20	7	22	10, 0	2	10
	21	9	25	15, 5	9	0
	15	15	26	11		5
	19	16	23	15		8
DP	25	40	16	85, 98	53	82
	25	54	21	59, 63	45	82
	22	42	28	51, 63		76
	20	42	35	85		78

IP = Phosphate determined directly i.e. inorganic phosphate + creatine phosphate + acetyl phosphate.

ATP = Phosphate determined after 7 minutes hydrolysis with N/1 HCl minus IP, i.e, chiefly adenosine triphosphate.

DP = Phosphate determined after 180 minutes hydrolysis with N/1 HCl minus (IP + ATP), i.e. chiefly hexose diphosphate.

exposure to mustard. Under the conditions of our experiments this symptom was just detectable on incubation following exposure of 5 minutes, and was maximal following exposures of 15–20 minutes. The first appearance of the symptom occurs after 5–6 hours of incubation; that is, after the onset of the inhibition of lactate utilization.

If there is a causal connection between these two phenomena, the inhibition of lactate utilization is the cause of the loosening, not the reverse.

(5) *Phosphate Fractions.* A study of the phosphate fractions showed a slight but definite increase in the total extractable phosphate (Tables V, VI). In a previous paper (1) it was noted that mechanical separation of the epithelium from the stroma also results in an increase in the extractable phosphate. One may raise the question whether the loosening of the corneal epithelium by mustard similarly activates a system for the mobilization of soluble phosphate.

DISCUSSION

The major effects of mustard injury disclosed in these experiments are an inhibition of the utilization of lactate and a decrease in the oxygen uptake, the decreased oxygen uptake being approximately equivalent to that required for the complete combustion of the unutilized lactate. These metabolic changes are found within the same dosage range as that required for the loosening of the corneal epithelium and occur in advance of the first noticeable manifestation of loosening. There is no proof that these two phenomena are causally connected. In the present discussion, we wish merely to analyze what interpretations would be imposed on our various findings by the acceptance of the notion that the metabolic changes which we have found, and the subsequent loosening of the corneal epithelium, are causally connected.

In the study of the loosening of the corneal epithelium it was shown that under anaerobiosis the loosening does not occur. Our present findings, that the oxygen uptake of mustard treated tissue is inhibited in proportion to the reduced consumption of lactate, present an apparent paradox for there appears, at first sight, to be no oxidation left over to account for the loosening of the epithelium. The onset of the decline in O_2 uptake, however, apparently lags behind the decline in lactate consumption by an interval of one or more hours. Since the time of onset of the inhibition of lactate utilization and of decreased O_2 uptake cannot be specified exactly, the magnitude of the lag between them cannot be estimated. The difference may, however,

be sufficient to account for the oxidation of appreciable cellular material. The fact that this excess of oxygen uptake over the apparently available substrate eventually stops may be taken to indicate that some oxidizable component finally gets used up in the process.

In the normal cornea after incubation for 15–20 hours the lactate reservoir is depleted, but the oxygen uptake in the normal tissue does not decline as it does after the utilization of lactate is inhibited by mustard. On the contrary, there is frequently a rise in O_2 uptake, and we have attributed this rise, and the absence of a fall, to the oxidation of non-carbohydrate metabolites. It may be noted that some partial loosening of the epithelium occurs under these conditions. Nevertheless one may well ask—how is the failure of utilization of such alternative substrates to be explained in the case of mustard injury? It may be supposed that the hydrogen transport system which links the oxidation of lactate to the reduction of molecular oxygen contains many steps. The failure of the system to oxidize alternative substrates when lactate cannot be utilized indicates that the inhibition produced by mustard may not affect specifically the lactic acid dehydrogenase, but rather some enzyme system higher up in the chain. On the other hand the fact that some oxidation (50–70% of normal) goes on even when the utilization of lactate is completely inhibited indicates that the hydrogen transport system for lactate and its alternates is a special one, distinct from that involved in the rest of the respiratory process. Glucose and glycogen are, for instance, utilized at a normal rate in mustard treated corneas, and their utilization is not associated with an extra accumulation of lactate except under anaerobiosis.

Our experiments, therefore, suggest that in the cornea there is a special hydrogen transport system for at least a major part of the lactate oxidation, that this transport system is damaged by mustard, and that, as a consequence, some component of this system, which normally is kept chiefly in its reduced state by the oxidation of lactate or its alternates, becomes oxidized. The hypothesis that these metabolic events are connected with a loosening of the epithelium requires the more specific assumption that the oxidation of this intermediate is the cause of the loosening, because on incubation under nitrogen the loosening does not occur.

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XII. FURTHER EXPERIMENTS ON CORNEAL METABOLISM IN RESPECT TO GLUCOSE AND LACTIC ACID*

HEINZ HERRMANN AND FAY H. HICKMAN

I. GLUCOSE CONSUMPTION AND LACTATE PRODUCTION

In preceding papers we have been dealing with some reactions of the endogenous carbohydrate metabolism in the excised surviving cornea. In particular, we followed the depletion of the stores of glycogen and of lactate, and observed that the lactate utilization is almost completely blocked by mustard within a short time after exposure.

There is only a small quantity of glucose in the fresh cornea and this amount disappears within the first two hours of incubation, while the stores of glycogen and of lactate are not exhausted for 12 and 20 hours respectively. It follows that in the case of the excised cornea the maintenance in the normal state for as long as one day, or the development of the pathological lesion (loosening of the epithelium) which does not appear before 5 hours after exposure to mustard, cannot be connected with the normal or disturbed metabolism of glucose. Although glucose metabolism could not, therefore, be of direct relevance for the maintenance and pathology of the excised cornea during this time interval, an examination of glucose utilization in the cornea was nevertheless desirable. In vivo, glucose is constantly supplied to the cornea by the tears and through the limbal capillaries. Moreover, our previous experiments have shown that externally supplied glucose is utilized, and that in the presence of glucose the lactate and glycogen reservoirs in the tissue are kept filled. Therefore, experiments were carried out in which the utilization of glucose was tested more directly both in the whole cornea and in the separated stroma, in normal samples as well as after exposure to mustard. By separating these experiments on the utilization of externally supplied glucose from our previous data on utilization endogenous materials, we hoped to gain some clarity in the presentation of the problem.

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

RESULTS AND COMMENTS

The experiments were carried out on beef corneas as described in the preceding papers. The anaerobic acid production of normal and mustard treated corneas was tested in the Warburg apparatus with and without added glucose after 20 hours of incubation of the tissue in a moist chamber at 29°C. At this time all endogenous stores of glucose have been completely exhausted and any effect of mustard on the metabolism should be fully developed. It was found (Table I) that in the absence of added glucose the anaerobic acid production of the controls and mustard treated tissues was the same, thus confirming

TABLE I

Manometric Determination of Anaerobic Acid Production by Beef Corneas after Preliminary Incubation

(20 hours at 29°C.)

CO_2 PRODUCTION IN CU.MM. PER CORNEA PER HOUR			
Controls	Controls + Glucose	Exposed	Exposed + Glucose
90	204	102	143
92	191	98	150
80	194	78	120
89	185	75	125
92	165	95	135
98	168	103	128
82	170	90	149
84	178	100	145
Av.....88	181	93	137

our previous conclusions that mustard does not affect the glycogenolytic processes in the tissue except for a slight acceleration of glycogen utilization during the first few hours after exposure. In the presence of added glucose there is a great increase in anaerobic acid production, but this increase is significantly less in the mustard treated tissue than in the controls. It is plain that a late effect of mustard is an injury to a part of the glucolytic mechanism. The experiments which follow show somewhat similar effect observable also under aerobic conditions.

In these experiments glucose was injected into the stroma of control and mustard treated corneas. The glucose and lactic acid content

were estimated chemically on some samples before incubation, on others after incubation for 10 hours (Table II). The glucose consumption of the control and mustard treated corneas was essentially equal, but the lactate reservoir rose to slightly higher levels in the controls than in the treated corneas. Since in the mustard treated corneas the utilization of lactate is, as we already know, markedly reduced, the difference in the rise of the lactate level is probably significant.

Similar experiments performed on the denuded stroma (Table III) showed that glucose consumption and lactate production ceases in the

TABLE II

Glucose Utilization and Lactate Production in Excised Corneas, Normally and after Exposure to Mustard

Incubation time: 10 hours at 32°C.
2 mgms. of Glucose per cornea. Two corneas per sample.

GLUCOSE CONTENT IN MICROGRAMS PER CORNEA			LACTATE CONTENT IN MICROGRAMS PER CORNEA		
Before incubation	After incubation		Before incubation	After incubation	
	Controls	Exposed		Controls	Exposed
1392	347	462	450	750	605
1419	351	523	420	620	530
1816	560	616	316	506	516
1780	490	530	328	483	466
Average.....1602	437	533	379	590	529
Glucose consumed.....	1165	1069	Increase in Lactate....	211	150
Consumed per hour....	117	107			

denuded tissue after 4 to 5 hours of incubation. During the first three hours the glucose consumption is about 100 micrograms per cornea per hour, practically equal to that of the whole cornea. Only about half of the glucose lost is recovered as lactate. No difference was found between controls and mustard treated tissues.

Since the stroma has no oxygen uptake, our failure to recover all the lost glucose as lactate required explanation. Studies were therefore made on the extractable phosphate of the tissue. Experiments previously reported have shown that, when whole corneas are incubated, no changes occur in the phosphate fractions determined on the epithe-

lium and stroma separately. It was found, however, that after incubation of the denuded stroma there is a very marked increase in the least easily hydrolizable phosphate fraction (mainly glycerophosphate) without any change in the total extractable phosphate. The increase occurs chiefly at the expense of inorganic phosphate which is almost completely used up in the process. The increase in slowly hydrolizable phosphate amounted to 20–30 micrograms phosphorus per

TABLE III

Glucose Utilization and Lactate Production in the Isolated Stroma with and without Exposure to Mustard

Exposure time: 15 minutes. Preliminary maintenance at room temperature. Incubation at 32°C.

PRELIMINARY MAINTENANCE	INCUBATION AFTER INJECTION	GLUCOSE UTILIZATION IN MICROGRAMS		LACTATE INCREASE IN MICROGRAMS	
		Control	Exposed	Control	Exposed
hrs.	*hrs.*				
3	3½	310	340	24	20
		380	280	124	119
4	4	410	400	80	80
		290	340	120	95
5	5	360	380	200	210
		550	580	205	220
2	8	430	415	210	230
		475	450	243	238
2	8	440	505	168	179
		450	390	205	212

cornea. If this is counted as glycerophosphate it accounts for the major part of the difference between glucose lost and lactate recovered. It appears, therefore, that the denuded stroma stops utilizing glucose because it is unable to transfer phosphate from glycerophosphate to glucose. This transfer requires the presence of carriers of energy-rich phosphate bonds. In the absence of the epithelium these carriers apparently are gradually lost. Presumably the epithelium normally assists the stroma in the production of such phosphate carriers.

In the intact mustard treated cornea no such marked change is found in the extractable phosphate fractions of the stroma. It is to be concluded, therefore, that the decrease in glucose consumption, which sometimes occurs after exposure to mustard, is probably not due to the same mechanism as that which occurs in the denuded stroma. It might be due to an inhibition of hexokinase, which Dixon (1) has shown to be specially susceptible to mustard injury. An examination of Table IV shows that the the mustard effect on glucose consumption does not occur regularly, but depends in large measure on the conditions of incubation following exposure to mustard. The fact that the loss of glucose consuming power in the denuded stroma is no more rapid

TABLE IV

Effect of Mustard upon Glucose Utilization under Varying Experimental Conditions

EFFECT OF MUSTARD ON GLUCOSE UTILIZATION	TISSUE	GAS PHASE	SUBSTRATE SOLUTION SUPPLIED
No inhibition	Whole Cornea	O_2	Injection of glucose in Ringer's
No inhibition	Whole Cornea	$N_2 + CO_2$	No external substrate suspended in Ringer + bicarbonate
No inhibition	Stroma	O_2 (no uptake)	Injection of glucose in Ringer's
Inhibition (30%)	Whole Cornea	$N_2 + CO_2$	Suspended in glucose solution in Ringer + bicarbonate

after mustard treatment than in the controls suggests that this mustard effect on the glucose metabolism may be located in the epithelium rather than in the stroma.

II. LACTIC ACID CONSUMPTION

In the experiments reported above it was found that the freshly denuded stroma can consume glucose at a rate of 100 micrograms per hour equal to that of the whole intact tissue. One might conclude that in the presence of a constant supply of glucose practically the whole carbohydrate requirement of the epithelium might be met by lactate supplied from the stroma, and that the epithelium consumes little or no glucose directly. This, however, seems not to be true, for if lactate

is injected into the stroma and the whole cornea incubated without added glucose, the lactate is consumed at a rate of 25 micrograms per hour; that is, at precisely the same rate at which the normal endogenous reservoir of lactate is depleted in the absence of added substrate. It is to be concluded that this is the maximum rate at which lactate can be transferred to or consumed by the epithelium. Since in the presence of abundant glucose the lactate reservoir rises only slightly, the stroma in the intact cornea cannot be utilizing glucose at a rate

TABLE V

Comparison of Disappearance of Endogenous and Injected Lactate in Control Corneas and after Exposure to Mustard

Exposure to mustard: 10 minutes.
Incubation: 10 hours at 32°C.
Three corneas per sample.

	MICROGRAMS OF LACTATE PER CORNEA		
		Average	Estimated Lactate Consumed
Before Incubation:			
Uninjected	373, 380	376	
Injected	1330, 1290	1310	
After Incubation:			
Controls:			
Uninjected	66, 105	86	290
Injected	1050, 1040	1045	265
Exposed to Mustard:			
Uninjected	386, 253	319	57
Injected	1253, 1200	1226	84

much greater than 25 micrograms per hour. When the epithelium is removed the rate of glucose utilization in the stroma increases threefold. Returning to the mustard problem, we found that exposure to mustard inhibits the utilization of injected lactate in the same degree as it inhibits the utilization of endogenous lactate (Table V).

Our previous analysis has shown that approximately three fourths of the lactate reservoir in the fresh cornea is to be found in the stroma. Though the amount in the epithelium represents a small fraction of the total, the concentration of lactate in the epithelium is about twice

that in the stroma. It is not clear whether the epithelial lactate is derived in whole or in part from the stroma supply, and whether part of the epithelial lactate reserve, which accumulates in the presence of an abundant supply of glucose, may be due to a bottle neck in the hydrogen transport system of the epithelium, which permits some lactate to be formed by glucolysis in excess of the rate of oxidation. The possible competition of various oxidizable substrates for a limited supply of oxidized coenzymes has already been discussed in a previous paper.

Distinct from the question of the origin of the epithelial lactate is the further question of the utilization of lactate by the epithelium. In order that the epithelium may utilize the stroma lactate, the transport of stroma lactate to epithelium, either by diffusion or by active transfer is required. Furthermore, the utilization of the stroma lactate by the epithelium may involve mechanisms distinct from those available for use of endogenous lactate within the epithelium. Since the utilization of lactate is inhibited by mustard it was possible to attack this question experimentally, and to test whether the utilization of lactate from the two sources is equally affected by mustard injury.

In a preliminary series of experiments the corneas, after exposure to mustard, were incubated for varying periods. The epithelium was then scraped off and the lactate content of the separated tissues estimated separately. The results were compared with fresh unincubated tissues and with control samples not exposed to mustard but incubated for the same period.

In control samples the epithelial lactate reservoir drops sharply during the first hour (Table VI). The stroma lactate remains unchanged during this period. Whether this is because no stroma lactate is transferred to the epithelium during this time, or because the rate of production of lactate from the rapidly exhausted endogenous glucose reserve in this tissue just equals the loss, is not clear. Since the fresh stroma contains glucose and under special experimental conditions has been shown capable of utilizing it, one would be inclined to assume that the latter is the case. About two hours after incubation a beginning depletion of the stroma lactate reserve becomes measurable. This coincides with the time of depletion of endogenous glucose. Also, it coincides with the time when the lactate concentration in the epithelium has fallen approximately to that in the stroma.

In mustard treated tissues the consumption of the epithelial lactate proceeds at an essentially normal rate during the first two hours, but with adequate dosage the utilization of stroma lactate is completely inhibited. It is not immediately clear whether the preliminary consumption of part of the epithelial lactate occurs because the mustard effect has not had time to develop, or because the epithelial mechanism for consumption of endogenous lactate is less sensitive to mustard injury. With more prolonged incubation the lactate content of the epithelium in mustard treated tissues declined no more rapidly than in controls, in spite of the fact that in the mustard treated samples no further supply of stroma lactate was available. It follows that the

TABLE VI

Disappearance of Lactate from the Epithelium of the Cornea

Incubation of whole corneas. Removal of Epithelium immediately before Lactate determination. Four corneas per sample.

LENGTH OF INCUBATION	LACTATE CONTENT OF THE EPITHELIUM IN MICROGRAMS PER CORNEA				AVERAGE
hours					
0	107	80	95		94
1½	63	54			59
2	60	41			51
3	36	35			36
4	40	32			36
5	31	26			29
10	7	17	12	2	10

mechanism for consumption of endogenous lactate in the epithelium is damaged by mustard, but, since some lactate consumption continues in the epithelium even when the utilization of stroma lactate is completely inhibited, it is apparent that the two systems are not equally sensitive to mustard injury.

In order to examine these matters in greater detail we have sought experimental conditions under which an appreciable time interval could be interposed between the exposure to mustard and the onset of utilization of the lactate stores. It is possible to achieve this by keeping the cornea at a lower temperature or under anaerobiosis for a preliminary period following exposure to mustard. At the end of this preliminary period samples are assayed for their content of lactate

in epithelium and stroma while other samples are incubated aerobically to test the loss of lactate in the two tissues. Neither of these procedures can be regarded as leaving the tissue unaffected except in preventing the consumption of lactate. As a matter of fact, both procedures had previously been shown to suppress the pathological process of loosening

TABLE VII

Lactate Disappearance in Corneal Epithelium after Preliminary Maintenance at 0°C.

INCUBATION AT 0°C.	INCUBATION AT 29°C.	LACTATE CONTENT OF EPITHELIUM IN MICROGRAMS PER CORNEA			
		Before Incubation at 29°C.		After Incubation at 29°C.	
		Control	Exposed to Mustard	Control	Exposed to Mustard
hours	*hours*				
7	3	115	105	55	51
				57	55
2½	2½	96	90	53	52
				53	51
7	3	72	67	42	42
				40	50
6	2	84	87	42	42
				45	52
6	2	84	93	47	54
				42	53
6	2	107	95	49	55
Average.....		93	89	48 (inhibition absent)	51
			LACTATE CONTENT OF WHOLE CORNEAS IN MICROGRAMS		
1	10	420	410	120	360
				190 (inhibition present)	330

of the epithelium and even to reduce slightly the loosening of the epithelium which occurs on subsequent aerobic incubation. This complication is, however, not without advantage, for if the loosening of the epithelium is, as we have suggested, connected with the inhibition of lactate consumption, then we should find that mustard treated tissues subjected to these preliminary treatments would show less

inhibition of lactate utilization on subsequent aerobic incubations than is the case in tissues incubated aerobically directly after exposure.

Preliminary incubation at zero degrees revealed no effect (Table VII). The decline of lactate concentration in the epithelium during the first 2 to 3 hours of subsequent incubation at 29°C was the same as in the controls, while estimates of the lactate content of the whole cornea following ten hours of incubation at 29°C. showed that the

TABLE VIII

Lactate Content in the Epithelium after Preliminary Anaerobic Maintenance

Incubation at 29°C.
Epithelium of 4 Corneas per sample.

INCUBATION ANAEROBICALLY	INCUBATION AEROBICALLY	LACTATE CONTENT OF EPITHELIUM IN MICROGRAMS PER CORNEA			
		Before Aerobic Incubation		After Aerobic Incubation	
		Control	Exposed to Mustard	Control	Exposed to Mustard
hours	*hours*				
2½	2½	74	88	32	40
				29	50
5	2½	101	122	69	86
				50	94
4	2½	92	125	59	62
				63	71
2½	2½	70	95	25	34
				34	45
4	2½	105	120	55	69
				63	77
4	2½	110	139	71	105
				67	103
Average.....		92	115	51	70
		Lactate Consumed		41	45

stroma stores were completely intact. This result might perhaps have been predicted, for at zero degrees both the biochemical consequences of the combination of mustard with the tissue and also any recovery from the injurious effects should be almost completely inhibited.

Experiments in which there was a preliminary period of anaerobic incubation, however, afforded quite clear cut results. In the first

place, there was a marked decrease in the inhibition of lactate consumption on subsequent aerobic incubation. In the second place no inhibition in the consumption of the epithelial lactate reservoir was found in those experiments (Table VIII), and all the remaining inhibition had to be attributed to an injury in the mechanisms for consumption of stroma lactate (Tables IX, X). These experiments considered by themselves leave it still unclear whether the mechanism for consumption of endogenous lactate in the epithelium is less susceptible to mustard injury than the mechanism for transfer and consumption of stroma lactate, or whether the former merely recovers more com-

TABLE IX

Disappearance of Lactate from Whole Corneas after Exposure to Mustard and after Preliminary Anaerobic Incubation at 15°C.

LACTATE CONTENT IN MICROGRAMS PER CORNEA			
After Anaerobic Incubation at 15°C. for 10 hours		Anaerobic Incubation at 15°C. for 10 hours followed by Aerobic Incubation at 28°C. for 10 hours	
Controls	Exposed to Mustard	Controls	Exposed to Mustard
300	396	8	160
316	342	25	174
271	270	26	166
266	415	25	150
Av....288	331	21	162
	Lactate Consumed	267	169

pletely under anaerobiosis. Taken in connection with the experiments without preliminary anaerobic incubation, the former of these two possibilities seems more likely, but in any case, it is clear from these experiments that the two mechanisms are sharply distinguished.

The consideration of these experiments reveals one further minor point. After anaerobic incubation, the lactate level in the epithelium rises somewhat higher in mustard treated corneas than in the controls. This may be correlated with the increased rate of glycogen consumption during the first few hours after exposure to mustard noted in a previous paper. It is also to be correlated with the diminished total

lactate production in the whole cornea after exposure to mustard in the presence of glucose. We have previously suggested that this deficit in lactate production is attributable to a partial inhibition of glucolysis in the epithelium. With a decrease in the ability of the epithelium to utilize its endogenous glucose, the mustard treated tissue would be expected to begin consuming its endogenous glycogen sooner than the controls, and this is what we have found. It is likely, however, that in the presence of extra glucose the utilization of glycogen in the epithelium is suppressed.

TABLE X

Lactate Disappearance from Whole Corneas after a Preliminary Anaerobic Maintenance Period

Anaerobic: 10 hours; Aerobic: 10–12 hours.
Incubation Temperature 29°C.

LACTATE CONTENT IN MICROGRAMS PER CORNEA			
After Anaerobic Maintenance		After Anaerobic and Subsequent Aerobic Maintenance	
Control	Exposed to Mustard	Control	Exposed to Mustard
1100	1180	690	690
950	920	690	810
1150	1250	590	760
1480	1150	720	810
740	1000	550	620
1000	1000	650	700
Av.....1070	1083	648	732
Lactate Consumed		422	351

SUMMARY

The experiments reported here have added somewhat to our knowledge of the metabolic interactions between corneal stroma and epithelium. It was found not merely that the epithelium consumes the lactate produced by the stroma, but indirect evidence suggests that in addition the rate of glucolysis in the stroma is markedly influenced by the presence of the epithelium. In other tissues, the intracellular control of the rate of glucolysis and glycolysis by the activity of the respiratory system is well recognized, and has been called the Pasteur

effect. The corneal epithelium exhibits a typical Pasteur effect in that glycogen is consumed more rapidly under anaerobic than under aerobic conditions. In addition, it appears that in the cornea the interaction of epithelium and stroma involves processes similar to those concerned in the Pasteur effect. In the intact tissue it is estimated that about one quarter of the carbohydrate requirement of the epithelium is furnished in the form of lactate by the stroma.

At least one of the metabolic interactions between stroma and epithelium is profoundly damaged by mustard injury in doses which likewise lead to a loss of cohesion between the two tissues. Moreover, anaerobic incubation which diminishes the effect of mustard injury in respect to loosening of the epithelium also diminishes the effect in respect to this metabolic interaction. It may be suggested, therefore, that the metabolic interaction between stroma and epithelium is connected with the mechanism for the maintenance of the tissue cohesion, and that injury to this mechanism by mustard results both in the loss of cohesion and in the loss of metabolic interactions.

The mechanism by which the epithelium consumes its endogenous stores of lactate is distinct from that by which it acquires and consumes the lactate of the stroma, the latter being more susceptible to mustard injury than the former. The question as to whether the transport of lactate from stroma to epithelium involves an active transfer or simple diffusion will be discussed in a subsequent paper.

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XIII. THE CONSUMPTION OF PYRUVATE, ACETOIN, ACETATE, AND BUTYRATE BY THE CORNEA*

HEINZ HERRMANN AND FAY H. HICKMAN

In the preceding papers we have been concerned with the utilization of glucose, glycogen, and lactate by the cornea. Presumably the major oxidative pathways for the consumption of all three of these substrates pass through pyruvate. Nevertheless no appreciable quantities of pyruvate (or other keto acids) are to be found in the cornea. One would expect, therefore, that the utilization of pyruvate must be very rapid. In the present paper we shall report the results of out studies on the utilization of pyruvate which, in certain respects confirm this expectation. The latter experiments were not pushed to a final conclusion since it was found that exposure to mustard yields no detectable change in pyruvate consumption. A partial picture of the route of pyruvate utilization in the cornea was nevertheless achieved and will be reported here.

TECHNIQUE

Beef corneas were used in these experiments. The manipulation of the corneas and the technique of application of inhibitors and metabolites has been described in previous papers. The method of assay of lactate has also been reported previously.

Pyruvate was prepared from a fresh sample of Eastman's pyruvic acid by fractional distillation, careful neutralization of the proper fraction with sodium hydroxide, and precipitation of the sodium salt with alcohol. Determinations of pyruvate in trichloracetic acid extracts of corneas were carried out according to Lu's method (1). Acetaldehyde was determined by the method of Stotz (2). CO_2 was measured manometrically as described below.

Acetate determinations were carried out as follows: 3 corneas were pooled for each sample and 8 ml. of N/12 H_2SO_4 and 1 ml. of a 10%

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

solution of sodium tungstate were added. The samples were allowed to stand for about one half an hour and then ground in a mortar for 15 minutes. The extracts were separated from the insoluble residue, and to each sample 10 ml. of a saturated solution of NaH_2PO_4, containing 1 ml. of concentrated phosphoric acid and 100 mg. of phenylhydrazine, were added. The mixture was placed in a vacuum still and the pressure reduced to 10–12 mm. Hg and steam slowly admitted. The connection of the pump was closed and the steam distillation continued for 20–30 minutes, the vacuum decreasing in this time to 50–60 mm. Hg. The distillate was titrated with 0.01 N NaOH using phenolphthalein as indicator.

Acetoin was determined by the method of Stotz and Rahberg (3). We used two corneas per sample. To these we added a sufficient amount of the tungstate sulfuric acid mixture to bring the volume to 10 ml. After standing at room temperature for at least 15 minutes the corneas were ground in a mortar and the solid particles removed by centrifugation. 2 ml. of the extract were used for conversion to diacetyl and half of this for distillation. 2 ml. of 2.5% bromine water were used for bromination. Octylalcohol, which is prescribed as a detergent to be used in the wash fluid, was not immediately available. We found that 0.02% zephiran[1] had the desired effect and did not interfere with the determination.

Butyric acid was determined by a method which followed in principle that of Bobbit and Deuel (4). Ten corneas were placed in 100 ml. of 0.1 N H_2SO_4 containing 1% sodium tungstate. After standing at room temperature for 15–20 minutes they were ground in a porcelain mortar without sand for at least 10 minutes. The fluid was poured into centrifuge tubes and 80 ml. of clear supernatant fluid was collected after centrifugation. This was transferred to an Erlenmeyer flask and 20 ml. of 10% $CuSO_4$ were added. Ten mg. of powdered $Ca(OH)_2$ were added and the suspension was shaken vigorously every 10 minutes for half an hour. After this the sample was filtered and 50 ml. of the filtrate used for distillation. 100 ml. of N/1 H_2SO_4 were added and distillation performed until only about 10 ml. remained in the distilling flask. The distillate was transferred to a flask connected with a

[1] Zephiran is alkyl (C_8—C_{18})-dimethyl-benzyl ammonium chlorid, Alba Pharm. Co.

reflux condensor. 100 ml. of van Slyke's mercury reagent were added to the distillate and the mixture was refluxed. When boiling commenced 10 ml. of a saturated potassium bichromate solution were added through the reflux condensor and the boiling was continued for 15 minutes. The fluid was then distilled off until 25–50 ml. remained

TABLE I

Utilization of Pyruvate by Beef Cornea

	AMOUNT OF PYRUVATE INJECTED	MICROGRAMS OF PYRUVATE RECOVERED PER CORNEA					
		Whole cornea			Stroma		
Immediately after injection	1 mg.	410 470	450 510	480 430	410 560	510	470
After incubation Time Temp.							
2 hr. 32°C.	1 mg.	210	210		490	480	
4 hr. 32°C.	1 mg.	125	115		370	390	
5 hr. 32°C.	1 mg.	100 120	105 110		390	480	
Immediately after injection	2 mg.	1535	1350				
After incubation Time Temp.							
4 hr. 29°C.	2 mg.	590	550				
Immediately after injection	4 mg.	3000					
After incubation Time Temp.							
2½ hr. 29°C.	4 mg.	1935	1930				

in the distilling flask. The distillate was aerated with CO_2 free air and titrated with 0.01 N NaOH using bromthymol blue as indicator.

RESULTS

1) *Pyruvate Consumption.* When sodium pyruvate is injected into the corneal stroma denuded of epithelium and tissue incubated practically all of the injected pyruvate can be recovered on subsequent extraction (Table I). It is not surprising that this tissue, which

has no measurable oxygen uptake, should fail to oxidize pyruvate, but it is rather remarkable that only small losses of pyruvate occur by dismutation or reduction.

When pyruvate is injected into the corneal stroma with the epithelium intact very rapid disappearance of pyruvate can be observed. This is particularly marked when 2 mg. or more of pyruvate is injected per cornea. With such a large surplus of substrate the rate of consumption is approximately 400 micrograms per hour per cornea. This is to be compared with the previously reported rates of consumption of glucose (100 micrograms per hour) and of glycogen and lactate (each 25 micrograms per hour). With lower concentrations of pyruvate in the tissue the rate of disappearance of pyruvate falls off roughly in proportion to the concentration. Since the pyruvate is consumed almost exclusively by the epithelium, one may compare the activity of this tissue with some others for which data are available in the literature. The wet weight of the epithelium of one cornea is approximately 80 mg. Since this is capable of consuming 400 micrograms per hour we reach a figure of about 5×10^{-2} mM per gram wet weight per hour. This is comparable to the rate found in heart muscle by D. H. Smyth (5).

The rate of pyruvate disappearance in these experiments is uninfluenced by anaerobiosis (Table II) or by exposure of the tissue to mustard (Table III), iodoacetate, fluoride, or arsenite (Table IV). The possible effect of mustard was tested for by injecting pyruvate into the cornea not only immediately after exposure to mustard but also after incubation periods of 4–8 hours following exposure. This was done in order to disclose the possibly delayed appearance of a mustard effect, but the consumption of pyruvate was normal in all these experiments.

2). *Lactate Production.* The fact that the rate of pyruvate disappearance was the same aerobically and anaerobically, the further fact that the aerobic disappearance of pyruvate was not associated with any increase in the oxygen uptake, both indicated that we were dealing with a dismutation rather than an oxidation of pyruvate. This conclusion was confirmed by the finding that approximately one half of the pyruvate which disappears can be recovered as lactate

TABLE II

Pyruvate Disappearance and Lactate Production under Anaerobic Conditions

Incubation time: 4 hours.
Temperature: 31°C.

	PYRUVATE ADDED	MICROGRAMS PER CORNEA					ESTIMATED PYRUVATE CONSUMED	ESTIMATED LACTATE PRODUCED FROM PYRUVATE
		Pyruvate		Lactate				
Before incubation	+	540	500	385	420			
Incubated under H_2	−			570	620	630		
Incubated aerobically	+	150	170	570	535		360	
Incubated in evacuated Thunberg tubes	+	150	130	880	890		380	280
Incubated under H_2	+	100	120	785	760		410	170

TABLE III

Disappearance of Pyruvate from Beef Cornea after Exposure to Mustard Vapor for 15 Minutes

Estimated uptake 0.6 micrograms mustard per cm² per minute.
Amount of pyruvate used for injection: 1 mg.
Corneas per sample: 2.
Incubation Temperature: 28°C.
Controls injected with pyruvate and incubated but not exposed to mustard.

INCUBATION TIME BETWEEN EXPOSURE TO MUSTARD AND INJECTION OF PYRUVATE	INCUBATION TIME AFTER INJECTION OF PYRUVATE	MICROGRAMS OF PYRUVATE RECOVERED PER CORNEA			
	None	Control	540	560	
4 hours	5 hours	Control	100	90	
		Treated	97	95	
12 hours	5 hours	Control	60	60	
		Treated	80	90	
5 hours	5 hours	Control	170	130	130
		Treated	170	160	145
8 hours	2 hours	Control	300	370	350
		Treated	350	360	310

(Table V). The rate of reaction as measured by the lactate production was also uninfluenced by anaerobiosis (Table II) or by exposure to mustard.

TABLE IV

Effect of Metabolic Inhibitors on Pyruvate Utilization

Incubation time: 4 hours.

	PYRUVATE CONTENT IN MICROGRAMS PER CORNEA	
Before incubation	1330	1330
Controls	630	500
Sodium arsenite, 0.01 M	700	610
0.02 M	790	900
Iodoacetate, 0.01 M	650	680
Before incubation	610	620
Controls	240	250
Fluoride, 0.02 M	240	180
Malonate, 0.025 M	240	180

TABLE V

Pyruvate Disappearance and Lactate Production

Amount of pyruvate used for injection: 2 mg. per cornea.
Incubation temperature: 29°C.

		PYRUVATE MICROGRAMS PER CORNEA	LACTATE MICROGRAMS PER CORNEA	ESTIMATED PYRUVATE CONSUMED MICROGM. PER CORNEA	ESTIMATED LACTATE PRODUCED FROM PYRUVATE MICROGM. PER CORNEA
Immediately after injection	No substrate added		315, 290, 350 250, 285		
	Pyruvate injected	1240, 1330	265, 275, 270 275, 285		
Incubation time					
2 hours	No substrate added		345, 320		
	Pyruvate injected	850, 1020	405, 425	350	117
4 hours	No substrate added		195, 220, 200 200, 190		
	Pyruvate injected	560, 530	505, 600, 440 430, 425	750	300

It must be remembered that our experiments were performed on whole corneas with their reserves of endogenous substrates intact. Under these circumstances the endogenous substrates compete for the

oxidation available to them through the hydrogen transport system. This hydrogen transport system appears to be operating at almost its maximum capacity in relation to the endogenous substrates, and not to be available for the very large amount of pyruvate which we supplied. Consequently, our finding that under the conditions of our experiment pyruvate disappears by dismutation, rather than by oxidation, does not imply that pyruvate is consumed in this tissue only by dismutative processes. On the contrary, the fact that the total O_2 uptake under normal circumstances closely matches the total carbohydrate consumption strongly suggests that pyruvate oxidation occurs in this tissue. In some preliminary experiments in which we have used corneal epithelium instead of whole corneas, an increase in O_2 uptake was found in the presence of pyruvate. It may well be that the partial inhibition after exposure to mustard, which we found in the consumption by the epithelium of its endogenous lactate (as distinguished from its consumption of stroma lactate), may be the result of an inhibition of pyruvate oxidase by mustard similar to that noted by Peters and Wakelin (6) in other tissues.

3) *CO_2 Production.* CO_2 was measured manometrically both aerobically and anaerobically. In each case the difference between the gas produced in the presence of pyruvate and in its absence amounted to about one mole CO_2 produced for each 4 moles pyruvate consumed. The general reaction can, therefore, be expressed approximately in the following equation:

$$4 \text{ Pyruvate} = 2 \text{ Lactate} + CO_2 + X$$

The aerobic experiment is shown in Table VI. Corneas were placed in bicarbonate buffer with air in the gas phase. In the absence of pyruvate the gas phase contracts on incubation (O_2 consumption minus CO_2 production). At the end of the incubation period HCl solution in the side arm is tipped into the main chamber liberating all the bicarbonate as CO_2. The difference in the total change in volume to the end of the experiment after addition of HCl, between controls and pyruvate containing vessels, is taken as the measure of the CO_2 produced from pyruvate. It is interesting that precisely the same difference in volume change is noted at the end of the incubation period, and that the same amount of CO_2 is liberated from the bicarbonate solution on addition of HCl in all samples. This can only

mean that in the dismutation of the pyruvate there is no change in the total number of fixed acid groups while one excess acid equivalent appears as CO_2. It follows that X in the equation includes two monobasic acid groups. If one were to take the above formula as exact, which probably it is not, the molecular formula of X would be equivalent to that of alpha-keto glutaric acid.

4) *Acetaldehyde and other Aldehydes.* No acetaldehyde could be recovered from control corneas and none accumulated during the consumption of large amounts of pyruvate. Tests for reducing sugars

TABLE VI

CO_2 and Lactate Production by Beef Corneas Incubated with and without Added Pyruvate

	CHANGE OF GAS VOL. IN CU.MM.	CHANGE OF GAS VOL. IN CU.MM. ON ADD. OF HCl		CHANGE OF LACTATE CONTENT IN MICROGRAMS	CHANGE OF PYRUVATE CONTENT IN MICROGRAMS
		Before Incubation	After Incubation		
No substrate added	−53	+72	+80	−60	
	−49		+87	−50	
2 mg. pyruvate added per cornea	+2	+84	+90	+260	−720
	+1		+98	+310	−700

ESTIMATED PYRUVATE UTILIZED	ESTIMATED LACTATE PRODUCED FROM PYRUVATE	ESTIMATED PYRUVATE USED TO YIELD CO_2
710	330	207

and for glycogen showed no differences between controls and pyruvate consuming samples.

5) *Acetate.* One dismutative reaction, the possible occurrence of which is well established, is represented by the following formula:

$$2\ \text{Pyruvate} = \text{Lactate} + \text{Acetate} + CO_2$$

Accordingly, we tested corneas for the presence of acetate. The method described above gave satisfactory recovery for a pure solution of acetate in amounts such as we had to expect in the corneal extracts. Additions of pyruvate and of lactate to the solution of acetate did not interfere with the recovery. The cornea itself does not contain significant amounts of volatile acids (Table VII), nor could any increase of volatile acid be detected on incubation for four hours at 29°C.

after injection of pyruvate, in spite of the fact that previous experiments showed that the greater part of the injected pyruvic acid disappears within this time. If only one tenth of the pyruvic acid which was injected into three corneas would have been transformed into

TABLE VII

Determinations of Acetate in Pure Solutions and in Corneal Extracts

	ml. .01 N NaOH
Acetic acid solution 1 ml	3.07
Recovery on distillation	2.78, 2.92
	2.80, 2.85
Acetic acid solution 0.5 ml	1.60
Recovery on distillation	1.52
Acetic acid solution 1 ml	2.72
Recovery on distillation	2.40
Recovery on distillation with 2 mg. Pyruvic acid added	2.59
8 mg. Lactic acid added	2.62
15 mg. Lactic acid added	2.55
Three Corneas per Samples	
Corneal extract	0.25, 0.34
Extract after injection of 4 mg. pyruvic acid per cornea	0.41, 0.38
Extract from cornea injected with 4 mg. pyruvate per cornea and incubation for 4 hours at 28°C	0.44, 0.32
Same extract after addition of acetic acid equivalent to 1.45 ml. .01 N NaOH	1.43
Recovery of acetate after injection into corneas immediately after injection	1.67, 1.90
	1.52, 1.62
	1.82, 1.98
Average	1.78
after incubation for hours at 28°C	1.74, 1.67
	1.37, 1.63
	1.45
Average	1.57

acetic acid, titration values of about 1 ml. of .01 N NaOH should have been obtained. In order to exclude the occurence of substances which might interfere with the distillation of acetic acid, an arbitrary amount of acetic acid was added to the extract of the incubated sample. About 70% of the added acetate could be recovered.

The absence of acetate after incubation of corneas with pyruvate could also be demonstrated with the lanthanum reagent of Krüger and Tschirch (7). Extracts from control corneas to which acetate was added gave clear positive reactions. Extracts from corneas which were incubated for four hours after injection of pyruvate gave negative results with the same reagent. From this series of experiments the formation of acetate from pyruvate seemed unlikely. However, we had still to exclude the possibility that acetate is utilized as rapidly as it is formed from pyruvate and therefore escapes detection. The rate of its disappearance was determined by comparing the amount recoverable immediately after injection with the amount recoverable after 4 hours of incubation. From the results listed in Table I, it can be seen that only a very small fraction of the injected acetate disappears, and if acetate would be formed in the course of the rapid disappearance of pyruvate it should accumulate in readily detectable amounts. The rate of consumption of acetate was 15 micrograms per cornea per hour.

6) *Acetoin.* Since acetate apparently is not formed from pyruvate, possibilities other than dismutation were examined. In the following experiments we investigated the formation and disappearance of acetoin. The characteristics common to the observed pyruvate disappearance in the cornea and the condensation of pyruvate to acetoin are the independence of O_2 supply, the production of CO_2, and the relative insensitivity to arsenite (8). On the other hand we had to postulate, in this case, an independent reaction in order to account for the appearance of lactic acid. After injection of pyruvate in amounts from two to four mg. per cornea and an incubation for 2–4 hours, no acetoin could be detected although acetoin, added to the same corneal extracts, could be recovered with a yield of 80%. However, acetoin itself disappears at a rapid rate from the cornea. After injection of 2 mg. per cornea about 0.4 mg. disappeared in three and a half hours, and after injection of 1 mg., per cornea 0.25 mg. was utilized in the same period (Table VIII). If it is taken into account that the formation of one molecule of acetoin (mol. weight 88) requires the condensation of two molecules of pyruvate (mol. weight 87), the higher value would correspond to a consumption of 800 micrograms of pyruvate.

This figure approaches our experimental data for the maximum pyruvate utilization (1000–1200 microgm. per cornea) and would indicate that acetoin disappears from the cornea almost as fast as pyruvate. Therefore, at the present time, acetoin formation cannot be excluded with certainty as a pathway of pyruvate utilization in the cornea, although no data are available as yet to provide positive evidence for this possibility.

TABLE VIII

Utilization of Acetoin

Incubation time: 4 hours.
Temperature: 29°C.

AMOUNT OF ACETOIN INJECTED MICROGRAMS PER CORNEA	AMOUNTS OF ACETOIN RECOVERED IN MICROGM. PER CORNEA				
	Before incubation	After incubation			
		Controls		Exposed for 15 min. to mustard vapor	
		recovered	utilized	recovered	utilized
2000	1250	900	350	750	500
	1200	900	300	700	500
	1350	850	500	750	600
	1400	1050	350	850	550
		950	450	800	600
	1400	1000	400	900	500
	1300	1050	250	1050	250
		1050	250		
1000	800	600	200	450	350
	900	650	250	550	350
Average.....			330		477

It was noted above that the disappearance of pyruvate is not inhibited by exposure to mustard. It was of interest, therefore, to determine whether the utilization of acetoin would prove equally insensitive to the application of mustard. The eyes were exposed to mustard vapor and then kept three and one half hours at room temperature in order to provide sufficient time for the reaction of mustard with the cell constituents. After this preliminary period the acetoin was injected and the corneas excised and incubated for 4 hours at 29°C.

From the results in Table VIII it can be seen that exposure to mustard does not inhibit the disappearance of acetoin. On the contrary, a slightly accelerated disappearance is indicated in the exposed corneas.

7) *Butyrate.* The tests on the utilization of butyric acid were carried out in order to obtain some information about the rate of

TABLE IX

Utilization of Butyrate

Butyrate used for injection: 500 micrograms per cornea.
Incubation temperature: 29°C.

INCUBATION TIME	RECOVERY OF BUTYRIC ACID IN MICROGM. PER CORNEA		
	Before incubation	Incubated controls	Incubated after exposure to mustard
10 hours	510	230	
	440	430	
	520	280	
		340	
20 hours	580	40	
	330	10	
		50	
		50	
	430	180	240
	290	50	210
	300		300
	360		240
	500	190	290
	480	250	260
	400	20	200
	390	50	80
	390	100	200
	290	170	
Average	400	100	220
Utilized		300	180

utilization of fatty acids in the cornea and to examine, if possible, the effect of exposure to mustard on the metabolism of a fatty acid, the determination of which is technically less disagreeable than the determination of acetic acid. The metabolism of butyrate is of additional interest since it is the metabolic precursor of the ketobodies

(acetoacetic acid, hydroxybutyric acid) which are metabolically very reactive substances. A possible connection with the preceding experiments arises out of the fact that acetoacetic acid is the product of one of the condensation reactions of pyruvate.

For the determination of butyric acid we used the procedure described above. The utilization of butyrate amounts to about 400 micrograms per cornea in 20 hours. This metabolic process is slow compared with pyruvate or acetoin utilization and about equal to the rate of consumption of lactate. Exposure to mustard has a marked inhibitory effect on the utilization of butyrate (Table IX). The question as to whether the utilization of butyrate is limited to the corneal epithelium as in the case of lactic acid has not been tested.

SUMMARY

The denuded corneal stroma consumes pyruvate at a very low rate, but when the epithelium is present pyruvate injected into the stroma is consumed at an extremely rapid rate. The consumption of pyruvate, in experiments on whole cornea, is uninfluenced by anaerobiosis or by exposure to mustard, iodoacetate, fluoride, or arsenite. About half of the pyruvate lost can be recovered as lactate, indicating that the process is at least in part a dismutation. For each 4 moles of pyruvate lost approximately 1 mole of CO_2 is recovered. The remainder is as yet unaccounted for. It may be ketoglutarate. It is not acetate, or acetaldehyde, or reducing sugar, or glycogen. Acetoin does not accumulate in the cornea during pyruvate consumption, but acetoin itself is consumed by the cornea at almost the same rate as pyruvate and its consumption is not inhibited by mustard. It cannot, therefore, be excluded as a possible product of pyruvate consumption. The consumption of butyrate was tested for comparison and found to be slow relative to pyruvate. Its consumption is inhibited by exposure to mustard.

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XIV. THE UTILIZATION OF RIBOSE AND OTHER PENTOSES BY THE CORNEA*

HEINZ HERRMANN AND FAY H. HICKMAN

The occurrence of ribose in nucleotides which act as the prosthetic group of enzymes, in coenzymes, and in nucleic acid, gives special importance to this substance, but information on the metabolic fate of ribose and other pentoses in animal tissues has advanced extremely slowly. The possibility of pentose formation from hexuronic acids, and of the condensation from triose and a C_2 precursor are discussed in recent reviews (1). The existence of a metabolic relation between uronic acids and pentoses is indicated by the finding that pentoses are excreted in the urine in higher concentrations in individuals producing excessive amounts of glucuronic acid, for instance, in the course of detoxification reactions (2). Lipmann (3) and Warburg (4) touched the problem of pentose metabolism in their investigations on the oxidation of phosphohexonic acid. The amount of oxygen consumed indicated that pentose was formed and that even 4 carbon and 3 carbon chains might appear as breakdown products. It should be recalled, however, that not ribose but arabinose is to be expected from the oxidation of the physiological hexoses. An inversion of one carbon atom in the hexose molecule would be required to yield ribose as an oxidation product. Dickens (5) studied the oxidation of pentose phosphates by Lebedew juice and also by animal tissues, and demonstrated the oxidation of ribose phosphate by red blood cells. Dische (6), who studied the metabolism of adenosine by laked red blood cells, obtained evidence suggesting the breakdown of the ribose part of the molecule into triosephosphate and a two carbon compound, possibly glycolaldehyde. More recent publications deal with attempts at elucidating the mechanism by which ribose is utilized for the synthesis of nucleotides (7). In the present paper we studied the disappearance of the ribose from the excised surviving cornea and tested the effect of various metabolic inhibitors and of mustard on this process.

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

EXPERIMENTS

1. *Methods.* The experiments were performed on beef corneas. The technique of maintenance of the corneas and of the injection of various substrates and inhibitors was the same as that described in previous papers. When ready for the chemical analysis, the tissue was ground in trichloracetic acid, centrifuged, and the supernatant fluid used for ribose determination.

Mejbaum's (8) modification of the Bial test was used to estimate pentose plus pentose nucleotide in the tissue extracts, the colorimetric reading being made with a Klett Summerson photoelectric colorimeter used with a #66 filter. Pentose standards containing 25 and 50 micrograms of ribose were carried along with each set of experiments. Glucose, fructose, ascorbic acid, and glucosamine interfere to such a slight extent with this colorimetric test under the prescribed conditions that their influence on the reading could be neglected. Glucuronic acid, however, produces a color of almost the same intensity as that which is obtained with pentose, since under the influence of hot, strong HCl as used in this test decarboxylation of glucuronic acid and formation of pentose may take place.

Estimates of injected ribose were also made with the reduction method (9) previously used for glucose determination. Standards and blanks were run along in all determinations.[1]

2. *Results.* Some material is extractable from the cornea which gives a positive Bial test. The amount is equivalent to about 200 micrograms ribose per cornea. One-third of this amount can be accounted for as adenosine triphosphate, and the rest is probably derived from other nucleotides. The greater part of this material is found in the epithelium (Table I). Since the corneal mucoid is confined to the stroma, the small stroma fraction in the Bial titration indicates that the interference of glucuronic acid in the determination is at most very small.

d-Ribose injected into the cornea disappears at an appreciable rate. In the experiments shown in Table II, 0.25 ml. containing 1 mg. d-ribose

[1] The pentoses used in this study were obtained from the following sources: d-Ribose, d-Xylose, and d-Lyxose were obtained from Pfanstiehl Chemical Co. Waukegan, Ill., and the d-Arabinose was obtained from Eastman Kodak Company, Chemical Division, Rochester, N. Y.

was injected into each beef cornea. About one-half of the injected fluid runs out of the needle holes immediately after the injection. This immediate mechanical loss is unavoidable in our experiments but by careful control of the injection technique can be kept reasonably uniform. The metabolic experiment, therefore, begins with 600—700 micrograms per cornea of which 200 is endogenous. After an incubation period of 20 hours at 32°C, the injected material has almost completely disappeared. The rate of disappearance of d-xylose and d-

TABLE I

Pentose (Bial Test) in Beef Cornea Calculated as Ribose

	MICROGRAMS PER CORNEA
Whole Cornea	163, 156, 214, 207
Epithelium	170, 176, 180, 174
Stroma	40, 23, 33, 44

TABLE II

Utilization of Injected Pentose by Beef Cornea during Incubation for 20 Hours at 32°C.

1 mg. injected.

MICROGRAMS PER CORNEA

d-Ribose		d-Xylose		d-Arabinose		d-Lyxose	
Before	After	Before	After	Before	After	Before	After
Incubation		Incubation		Incubation		Incubation	
734	360	651	309	660	615	705	510
582	222		423	654	519		495
603	300		313	564	525		556
726	320		383	630			593
673	336		296	531			

ribose is much greater than that of d-lyxose and d-arabinose. This difference may be due either to a difference in the actual rate of metabolic breakdown or to a difference in the rates with which the various pentoses penetrate into the epithelium.

The corneas into which ribose has been injected preserve their stores of glycogen (Table III) in the same way as do corneas injected with glucose solution. There is also a marked inhibition of lactate disappearance (Table IV).

The rate of disappearance of ribose in the presence of various metabolic inhibitors was examined. Iodoacetate exerts a marked inhibitory effect. The threshold for iodoacetate inhibition of glucose utilization is, however, still lower. Thus with 0.3 mg. of iodoacetate per cornea

TABLE III

Effect of Injection of Ribose and Glucose on the Glycogen Content of the Cornea after Incubation for 20 Hours at 32°C.

1 mg. sugars injected.

CONTROL	GLYCOGEN CONTENT IN MICROGRAMS PER CORNEA	
	Ribose	Glucose
600	908	933
575	756	1075
	1002	

TABLE IV

Effect of Injection of Ribose on the Lactic Acid Content of the Cornea after Incubation for 12 Hours at 32°C.

1 mg. Ribose injected.

LACTIC ACID CONTENT IN MICROGRAMS PER CORNEA	
Without Ribose	With Ribose injected
123	273
140	296
113	273
80	250
116	280
130	213
60	160
26	236
Average.....99	247

(about 2×10^{-3} M), glucose utilization is diminished by 70% but ribose utilization by only 30% (Table V).

In table V experiments are listed in which ribose disappearance was followed both by the Bial test and by ferricyanide reduction. The effect of iodoacetate in inhibiting the utilization of ribose is apparently stronger in respect to reducing power of the injected substrate than in

respect to the Bial test. Thus, by the application of iodoacetate, two steps in the metabolic breakdown of ribose become discernible. The first step results in the formation of a product which fails to give the Bial reaction but still has reducing capacity. In the second step re-

TABLE V

Comparison of the Effect of Iodoacetate on the Utilization of Ribose and Glucose

	MICROGRAMS OF SUGAR PER CORNEA				
	Before Incubation	After Incubation 20 Hours at 32C°			
IODOACETATE INJECTED	0	0	0.3 mg.	0.5 mg.	1 mg.
Ribose Bial Test	700	294 276	396	430 400	438
	699	270 294	350	386 566	523
	783	353 282	248	366 565	570
	736	380 303	403	666 438	456
	889	290 260	423	626	566
	696	316 313		366	
		320			
Average	751	304	364	480	511
Ribose Ferricyanide Test	823	340 266	386	576 623	666
	873	326 262	466	546 611	716
	733	335 320	473	513 654	713
	693	226 300	466	693 726	
Average	781	297	448	618	698
Glucose Ferricyanide Test	853	285	678	720	
	800	280	613	766	
	826	262	604		
	853	205	706		
		306			
		326			
Average	833	277	650	743	

ducing capacity is lost. Iodoacetate apparently inhibits the second step more strongly than the first. Since even the disappearance of reducing capacity is less sensitive to iodoacetate inhibition in the case of ribose than in the case of glucose, it would seem improbable that the reducing substance derived from ribose is triosephosphate. Dische

(6) has described a breakdown product of ribose which has reducing power, and it may be that this is the intermediate which is formed in the

TABLE VI

Effect of Various Metabolic Inhibitors on the Utilization of Ribose

1 mg. Ribose injected.

AMOUNT INHIBITOR INJECTED PER CORNEA		MICROGRAMS OF RIBOSE PER CORNEA DETERMINED AFTER INCUBATION FOR 20 HOURS AT 32°C.	
		Inhibitor Added	Control
Fluoride	1.0 mg.	633 573 603	290 333
	0.3 mg.	383 303 336	326
	0.1 mg.	300 350 193	233
Arsenite	1.0 mg.	360 350 373	193 270 193
	0.5 mg.	273 285	276 210
Malonate	1.0 mg.	290 243 320	293 250 276

TABLE VII

Effect of Treatment with Mustard on the Utilization of Ribose by the Cornea during Incubation for 20 Hours at 32°C.

1 mg. Ribose injected.

EXPOSURE TIME IN MINUTES	MICROGRAMS RIBOSE PER CORNEA	
	Control	Treated
10	249	261
	203	243
	210	282
	240	276
20	207	369
	213	295
	240	345
30	390	483
	346	490
	270	343
	286	362

cornea. Of the other metabolic inhibitors, tested fluoride gives a marked inhibition of ribose utilization, arsenite has a relatively small effect, and malonate is without influence (Table VI).

The effect of exposure of the cornea to mustard on the subsequent utilization of ribose is very small (Table VII). Corneas which had been exposed to mustard vapor at room temperature for 10 minutes[2] showed a normal rate of disappearance of ribose. Exposure for 20—30 minutes resulted in a slight decrease in the rate of disappearance of ribose.

In the small inhibition of ribose utilization which was observed after prolonged exposure to mustard vapor, no discrepancy was found between the results of the Bial test and of ferricyanide reduction, indicating that mustard does not preferentially inhibit the second step in the breakdown as does iodoacetate.

DISCUSSION AND SUMMARY

Our experiments show a slow but steady utilization of d-ribose and d-xylose by corneal tissue. The rate of disappearance is about 10—12 micrograms per hour per cornea, which is about one-tenth that of the rate of utilization of glucose and one-half that of lactate and glycogen.

d-Lyxose is utilized only at half the rate of d-ribose, and with d-arabinose no disappearance could be measured with certainty. High selectivity of cells in respect to utilization of pentoses has become known in case of the absorption of pentoses from intestine (10) and the metabolic utilization by various bacteria (11). In our case it remains undecided as to whether the observed difference in the utilization of pentoses by the cornea rests in the specificity of the permeability or in the specificity of the effective enzymes.

In the pathway of ribose utilization two steps became discernible by the use of iodoacetate. As pointed out above, at a proper concentration of this metabolic inhibitor, the first step in the ribose breakdown takes place at a slightly diminished rate only, while a subsequent reaction is greatly inhibited, leading to an accumulation of a breakdown product with reducing properties. During an incubation for 20 hours, an amount of this intermediary accumulated which corresponds to 150 micrograms of ribose per cornea. With experiments on a larger scale it should be possible to obtain sufficient material to identify this intermediary.

[2] Under the conditions of these experiments the estimated uptake of mustard was 0.6 micrograms per cm^2 per minute.

We have no indication as yet as to whether the breakdown of ribose in the cornea involves phosphorylation. The fact that the utilization of ribose has been observed with highly viable and intact cells, and yet under conditions which allow complete analysis, gives some promise that syntheses with ribose, e.g. nucleotide formation, could be observed with this tissue.

The utilization of ribose is markedly inhibited by iodoacetate and fluoride, but is only slightly inhibited by mustard. In the presence of ribose the utilization of glycogen and of the lactate depot in the cornea is suppressed.

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XV. STUDIES ON NON-PROTEIN NITROGEN IN THE CORNEA*

HEINZ HERRMANN AND SYLVIA G. MOSES

The present studies were undertaken in preparation for the more ambitious project of investigating the nitrogen metabolism of the cornea with the aid of isotopically labelled compounds. When the work had to be abandoned on account of technical difficulties, it was still found possible to study the production and utilization of some nitrogenous substances in the cornea for which suitable microanalytic methods were available.

TECHNIQUE

The experiments were performed on beef corneas. The preparation, exposure to mustard,[1] and incubation of corneas have been described in previous papers. Only uninjured and perfectly clear corneas were used in these experiments. Corneas were selected carefully with regard to uniformity of size in each experiment. After incubation the corneas were vigorously ground for 10 minutes, with addition of sand, in 5% trichloracetic acid, two corneas being used for each sample. The extract was poured into a filter and the extraction repeated twice with 3 ml. and 2 ml. of trichloracetic acid solution, grinding the tissue each time for two minutes. The combined filtered extracts were made up to 10 ml., one half of which was used for ammonia determination, the other half for NPN determination. The portions used for ammonia determination were alkalinized with one drop of 40% NaOH and 2.5 ml. of a saturated solution of potassium carbonate. A gentle stream of air carried the ammonia into a receiver containing 5 ml. of half saturated boric acid to which brom-cresyl-green was added as indicator. The ammonia was then titrated with N/100 H_2SO_4. The results are tabulated below as ammonia, but in work with tissues it should be

* The work described in this paper was done in part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

[1] Under the conditions of exposure used in these experiments it was estimated that 0.6 micrograms of mustard were taken up per cm^2 per cornea per minute.

kept in mind that by this method volatile amines are determined as well.

Determinations of amino nitrogen were carried out according to the method of Peters and van Slyke (1). In preparing the extracts in this series of determinations we avoided the tedious manual grinding of the tough corneal tissue by using the mechanical extractor which has been described elsewhere (2). The trichloracetic acid extracts of the corneas obtained with this instrument were filtered and evaporated to dryness in order to remove the trichloracetic acid. The residue was dissolved in 10 ml. of distilled water and a 5 ml. portion was used for amino nitrogen determinations. The second portion was used for determination of total NPN by microkjeldahl.

For the determination of serine, three corneas were pooled for each sample when whole corneas were examined, and 6 to 9 corneas for the determinations on separated epithelium or stroma. Those corneas injected with dl serine received 2.0 mg. in 0.25 ml. of 0.3% saline. Unless otherwise indicated, the corneas were incubated at 38°C for approximately 10 hours. The tissues were extracted in 5% trichloracetic acid, the extracts were neutralized, and serine determinations performed on the whole extract according to the procedure of Boyd and Logan (3) as follows: The samples of approximately 15 ml. were transferred to a 300 ml. Kjeldahl flask and 3 drops of methyl red and 4 ml. of 25% potassium arsenite added. Approximately 3.0 ml. of 0.5 M periodic acid was required to adjust the reaction mixture to the proper pH. The formaldehyde was distilled off and determined colorimetrically. For the analysis of samples containing less than 1.0 mg. serine, 2.5 ml. aliquots of the distillate were put into test tubes calibrated at 25 ml., 0.25 ml. of the purified chromotropic acid reagent were added, and the volume made up to 8.5 ml. with distilled water. The tubes were cooled in an ice bath and 5.0 ml. of concentrated sulfuric acid added slowly with constant shaking. The mixtures were allowed to cool to the temperature of the ice bath and then made up to 25 ml. with concentrated sulfuric acid. They were placed in a boiling water bath for ten minutes, cooled and read with a # 56 filter in a Klett Summerson photoelectric colorimeter.

The specificity of the test is not absolute. It rests upon the oxidation of serine to formaldehyde by periodic acid and the subsequent

colorimetric determination of the formaldehyde. The reaction for formaldehyde is quite specific, but there are potentially in the tissue some substances, other than serine, which will yield formaldehyde on oxidation with periodic acid, and hence will be titrated as serine. Determinations of endogenous serine by this method are, therefore, somewhat equivocal. We undertook measurements on the endogenous serine, or more properly the endogenous formaldehyde producing substances, essentially as controls for subsequent experiments in which much larger amounts of serine were injected into the tissue. In these experiments the small fraction of endogenous formaldehyde producing substances was negligible.

(1) *Non-Protein Nitrogen.* The determination of the non-protein nitrogen (NPN) level in the excised cornea was carried out for several reasons. The data were needed as a basis of experiments on utilization of externally supplied nitrogen, which made it necessary to find out whether during incubation the level in the whole cornea and in the stroma would remain constant or would show a definite change. Possible small changes in the NPN level seemed of interest also in connection with the mechanism of the loss of adhesion of the epithelium. We observed that among the enzymes tested only trypsin and chymotrypsin caused a loosening of the corneal epithelium and a similar observation was made by Medawar (4) on skin. Therefore, it seemed possible that proteolytic processes, detectable by changes of the NPN level, might play a role in the loosening. In the case of vesication caused by thermal burns (5) and by mustard (6), some connection with proteolytic processes has been suggested by Peters and his coworkers.

Results. The normal beef cornea contains about 250 micrograms of extractable non-protein nitrogen, of which slightly less than 10% is recovered as ammonia. About half of the total nitrogen and about 80% of the ammonia is in the stroma. Since the wet weight of the epithelium is about one tenth that of the stroma, the concentration of NPN in the epithelium is about 5 times that in the stroma, while the concentration of ammonia in the two tissues is more nearly equal.

On incubation either of the whole cornea or of the isolated stroma, the ammonia content remains fairly constant. This is true both for controls and for tissues previously exposed to mustard.

On incubation either of the whole cornea or of the isolated stroma,

only very small changes were observed in the NPN content in normal controls, and the direction of the change was inconsistent. If, however, the whole cornea was incubated for 3 hours or more after exposure

TABLE I

Ammonia and Total Non-Protein-Nitrogen Content of the Whole Cornea and of the Isolated Stroma after Exposure to Mustard

Exposure time to Mustard: 15 minutes.
Incubation: 9½–10 hours at 28–32°C.

MICROGRAMS NITROGEN PER CORNEA

Ammonia Nitrogen			Total Extractable Non-Protein-Nitrogen		
Before Incubation	After Incubation		Before Incubation	After Incubation	
Control	Control	Exposed to Mustard	Control	Control	Exposed to Mustard
Whole Cornea					
20.8	21.5	20.5	249.5	235.5	323.0
15.8	14.3	13.6	229.7	248.5	274.2
13.6	12.9	15.8	240.0	228.0	304.0
15.2	20.0	21.5	196.3	223.9	277.0
18.4	21.5	17.9	264.8	231.8	290.3
			270.0	226.3	254.5
			272.3	284.7	337.8
			255.0	259.3	303.0
			229.9	251.0	289.8
			183.0	203.0	272.8
			272.0	257.0	301.0
Av.....16.7	18.0	17.8	242.1	240.8	292.5
Isolated Stroma					
18.0	14.3	15.1	132.8	130.4	138.0
12.0	11.6	11.6	128.0	135.0	125.0
14.1	12.9	19.3	134.2	123.8	136.4
14.4	12.9	14.4	123.6	107.5	106.0
Av.....14.6	12.9	15.1	129.7	125.1	126.4

to mustard there was a regular increase in NPN of 10—20%. This increase after exposure to mustard was not observed if only the isolated stroma was incubated (Table I).

After exposure to mustard and incubation of whole corneas for two hours no increased NPN was observed, but after three hours of incu-

bation the greater part of the excess NPN was already present. Some additional increase was found on incubation up to 10 hours,

TABLE II

Total Non-Protein-Nitrogen after Varying Lengths of Incubation

Exposure time to Mustard: 15 minutes.
Incubation temperature: 28°C.

Incubation Time	Micrograms nitrogen per cornea			
	Before Incubation	After Incubation		
	Control	A Control	B Exposed to Mustard	Difference B-A
2 hrs.		262.0	261.0	−1
		257.5	261.1	+3.5
3 hrs.		249.5	282.0	32.5
		246.5	281.5	25.0
4 hrs.		254.5	264.5	10.0
	250.0	268.0	279.0	11.0
		255.0	299.0	44.0
		261.0	287.0	26.0
5 hrs.		251.0	283.0	32.0
		264.5	284.0	19.5
6 hrs.	265.3	272.5	314.7	42.2
	250.0	258.0	301.5	43.5
		262.0	292.8	30.8
10 hrs.		259.3	303.0	43.7
		245.5	313.0	67.5
		260.5	318.0	57.5
		236.3	290.5	54.2
		263.0	307.0	44.0
		284.7	337.8	53.1
20 hrs.	224.0	217.0	276.0	59.0
		247.0	304.0	57.0
		256.0	324.0	68.0
	243.0	235.5	323.0	87.5

but prolonged incubation up to 20 hours yielded but a very slight increase in NPN (Table II).

The full effect in respect to NPN increase is produced by 15 minutes

exposure to mustard vapor at room temperature. Increase of the exposure time up to 90 minutes yielded no greater excess of NPN. When the exposure time was reduced to 5 minutes the yield of increased NPN was reduced to about half (Table III).

TABLE III

Total Non-Protein-Nitrogen after Varying Lengths of Exposure to Mustard

Incubation: 10 hours at 28°C.

	MICROGRAMS NITROGEN PER CORNEA				
Length of Exposure	Before Incubation	After Incubation			
	Control	A Control	B Exposed to Mustard	Difference B-A	Average Difference
5 min.		263.0	277.0	14.0	
		236.3	258.0	21.7	
		260.5	282.0	21.5	
		245.5	295.0	49.5	26.7
10 min.		236.3	274.0	38.0	
		263.0	285.5	22.5	
		268.0	313.0	45.0	35.2
15 min.		259.3	303.0	43.7	
		245.5	313.0	67.5	
		260.5	318.0	57.5	
		236.3	290.5	54.2	
		263.0	307.0	44.0	
		284.7	337.8	53.1	53.3
60 min.	275.3	284.7	339.0	54.3	
	255.0	259.3	307.3	48.0	
	229.8	251.0	272.0	21.0	37.8
90 min.	183.0	203.0	248.0	45.0	
	275.0	257.0	310.0	53.0	
		268.0	299.0	31.0	43.0

When the temperature of incubation was varied between 28°C. and 35°C., no change in yield of excess NPN was found. Incubation at 15°C to 18°C resulted in only about half as much excess NPN (Table IV).

Further analysis showed that the amino nitrogen fraction of the

total NPN also increases after exposure to mustard. The increase is small but statistically significant. The increase of the amino N amounts to one half to two thirds of the corresponding figures for the total NPN. Since 30% of the amino acids which constitute the cell

TABLE IV

Total Non-Protein-Nitrogen after Varying Incubation Temperature

Exposure time to Mustard: 15 minutes.
Incubation time: 9 to 10 hours.

	MICROGRAMS NITROGEN PER CORNEA				
Incubation Temperature	Before Incubation	After Incubation			
	Control	A Control	B Exposed to Mustard	Difference B-A	Average Difference
15–18°C.	251.0	234.0	253.0	19.0	
	281.0	238.6	267.4	28.8	
	233.0	230.7	248.7	18.0	
	251.7	259.3	280.8	21.5	
	275.0	262.0	280.6	18.6	21.2
23°C.	271.0	251.0	280.5	28.5	28.5
28°C.	229.0	251.0	289.8	38.8	
	183.0	203.0	272.8	69.8	
	272.0	257.0	301.0	44.0	
		245.5	313.0	67.5	
		260.5	318.0	57.5	
		236.3	290.5	54.2	
		263.0	317.0	44.0	53.7
33–35°C.	243.1	244.9	291.0	46.1	
	256.0	269.0	299.8	30.8	
	281.0	244.0	305.4	61.4	
	191.0	202.0	250.6	48.6	46.7

proteins contain nitrogen in a form which is not determined by the van Slyke manometric method, and since the proteolysis does not necessarily proceed to completion, these figures would seem to indicate that the entire increase in NPN is due to proteolysis (Table V).

(2) *Fractionation of Epithelial Proteins.* In an attempt to find a clue for an explanation of this limited increase in the NPN level, it

seemed of interest to examine the corneal epithelium for changes in the state of the proteins. We measured the amount of protein extractable from normal and mustard treated corneas in various electrolyte solutions: 22% Na_2SO_4, 10% NaCl, and 10% NaCl followed by N/1

TABLE V

Effect of Mustard on Total Non-Protein-Nitrogen and Amino-Nitrogen Content of Beef Corneas

Exposure to Mustard: 15 minutes.
Incubation: 10 hours at 28°C.

MICROGRAMS PER CORNEA

Total NPN		Amino-N	
Controls	Exposed	Controls	Exposed
303	393	105	114
305	355	85	117
298	337	102	104
311	310	115	107
330	311	114	123
310	324	100	126
294	343	105	120
288	359	109	121
333	339	84	133
300	334	98	142
231	333	99	159
294	369	81	112
291	313	95	104
309	351	98	114
315	332	99	120
297	285	103	133
271	312	93	117
	307	81	133
		109	
Av.....299	334	99	121
Excess NPN.....	35	Excess Amino-N.....	22

$NaHCO_3$. The time of exposure to mustard was 15 minutes followed by incubation at 29°C for 5–7 hours. During the extractions all materials were kept in the refrigerator at 5°C.

For the extraction with 22% solution of Na_2SO_4 six corneas were pooled in each sample and the extraction was extended over a period of

48 hours, replacing the first 50 ml. portion of the extraction fluid after 24 hours. To the collected extract (total 100 ml.) we added 20 ml. of 50% trichloracetic acid and allowed it to stand for 12 hours. The precipitate was collected by centrifugation, the supernatant was discarded and the precipitate dissolved in concentrated H_2SO_4. An aliquot was used for determination of nitrogen.

In several experiments samples containing three corneas were first extracted with 10% NaCl solution for two 24 hour periods using 10 ml. and 5 ml. portions which were collected by decantation. At the end of the second day the epithelium became so loose that it could easily be removed in sheets with a forceps. The separated epithelium was transferred to 12 ml. centrifuge tubes and the extraction of the separated epithelium was continued, daily adding a fresh 2 ml. portion of the 10% NaCl solution for five more times, and separating the extracts by centrifugation. The collected extracts were precipitated with 20% trichloracetic acid. In one series the extracts were saturated with $MgSO_4$. After 12 hours a small precipitate developed which was filtered off. The collected precipitate was dissolved by washing the filter with several portions of physiological saline until no further protein came into solution. Aliquots of the pooled washings were used for nitrogen determinations. In another series in which the corneas had been extracted with 10% NaCl we continued the extraction of the residues for three days with 2 ml. portions of a N/1 solution of $NaHCO_3$. These extracts were also precipitated with trichloracetic acid and the precipitate dissolved in concentrated H_2SO_4 for N determination.

Results. The extraction with Na_2SO_4 and with $NaHCO_3$ yielded very small amounts of protein. A considerable yield was obtained on extraction with 10% NaCl solution. We noticed, however, that the yield of proteins obtained in the first two days of the extraction of the whole intact cornea was very small. The bulk of the precipitable material was extracted from the isolated epithelium in the first two days following the separation. The assumption that most of the protein in our extracts is derived from epithelial cells is corroborated by the fact that the sum of the proteins in the extracts plus the protein in the extracted epithelial residue agrees closely with the amount of total protein calculated from the average freshweight of the epithelium

(about 12 mg. protein per cornea). Comparing the figures for the amount of protein extracted with 10% NaCl, and for the protein in the epithelial residue, we find that more than half of the proteins of the epithelial cells are extracted.

No significant differences could be observed between the fractions which were obtained from normal corneas and from corneas after exposure to mustard (Table VI). However, it should be pointed out that

TABLE VI

Protein Content of Extracts of Normal Corneas and of Corneas after Exposure to Mustard

Exposure to Mustard: 15 minutes.
Incubation: 5–7 hours at 29°C.
The figures give mg. protein (mg. nitrogen × 6.25) extracted per cornea.

EXTRACTION WITH 22% Na_2SO_4 PRECIPITATION WITH TRICHLORACETIC ACID										AVERAGES
Controls	2.0	2.1								2.05
Exposed	1.9	2.1								2.0
EXTRACTION WITH 10% NaCl, PRECIPITATION WITH TRICHLORACETIC ACID										
Controls	8.0	7.8	8.0	7.2	9.5	8.6	8.8	7.1	7.8	8.1
Exposed	8.0	8.0	7.1	8.0	8.3	9.6	8.0	7.7	7.4	8.0
EXTRACTION WITH 10% NaCl, PRECIPITATION BY SATURATION WITH $MgSO_4$										
Controls	0.8	0.8								0.8
Exposed	0.7	0.8								0.85
EXTRACTION WITH $NaHCO_3$ AFTER PRELIMINARY EXTRACTION WITH 10% NaCl PRECIPITATION WITH TRICHLORACETIC ACID										
Controls	0.8	0.6								0.7
Exposed	0.7	0.7								0.7
Residue after extraction with 10% NaCl										6.0

previous calculations indicated that the observed increase in non-protein nitrogen would correspond to the proteolysis of not more than 1% of the total protein in the epithelium. It would be necessary to devise finer methods of fractionation or to carry out larger series of experiments to detect differences of this order of magnitude. An experiment in which we attempted to measure the adsorption of trypsin on the surface of corneas likewise failed to show any difference between mustard exposed tissues and controls, perhaps also because of inadequate sensitivity of the method.

(3) *Utilization of Ammonia.* Injected ammonia (supplied as ammonium succinate) is utilized at a rate of about 5 micrograms per hour per cornea (Table VII). Simultaneous determinations of the total non-protein nitrogen (NPN) revealed a loss equivalent to the disappearance of the ammonia, indicating that ammonia or an equivalent amount of some other NPN factor is synthesized into a non-extractable constituent of the cornea. This observation gives increased significance to the results which we obtained on examination of the effect of

TABLE VII

Utilization of Ammonia Nitrogen in Beef Corneas on Incubation

Incubation: 10 hours at 28°C.
Ammonium succinate injected: about 200 micrograms N per cornea.

MICROGRAMS PER CORNEA							
Ammonia Nitrogen				Total NPN			
Control		Ammonium succinate injected		Control		Ammonium succinate injected	
Before	After	Before	After	Before	After	Before	After
Incubation		Incubation		Incubation		Incubation	
20.8	24.5	143.5	100.8	277.5	258	418	315
21.5	27.3	141.5	86	287.5	231.5	405.5	296
28	35.8	145.5	83.1	252	232.3	384.8	266.3
33	33.7	158.6	105.4	301.6	272	486	401
19.8	20.8			303.6	222.6		
				270	226.3		
				264.8	261.8		
Av...24.6	28.4	147.3	93.8	279.6	243.5	423.6	319.6
Change +3.8		−53.5		−36.1		−104	

mustard on the ammonia utilization. In these experiments we found no inhibition of the disappearance of ammonia after an exposure of the corneas for 15 minutes to mustard vapor (Table VIII). These negative results demonstrate that some synthetic processes in the tissue are not interfered with by a dose of mustard which causes severe clinical symptoms in the cornea in vivo, and is accompanied by a definite damage to the epithelial cells in the excised tissue and inhibits certain metabolic reactions such as lactate disappearance.

Comparing the averaged figures given here with those in Table I

some divergence will be noticed. In these experiments a slight decrease of the NPN in the incubated control corneas was observed while in the previous series of experiments no significant change could be detected. It is possible that these are real differences which occur in different batches of corneas. It should be pointed out, however, that the present series of experiments include a smaller number of samples and that a deviation of the average value could be due to the wide scattering of the individual samples. Also, it should be pointed out that the control corneas in this series of experiments were injected with saline solution, whereas the corneas in the previous experiments were

TABLE VIII

Effect of Mustard on the Utilization of Ammonia Nitrogen

Ammonium succinate injected: about 200 micrograms N per cornea.
Incubation: 10 hours at 28°C.
Exposure time to Mustard: 15 minutes.

AMMONIA NITROGEN IN MICROGRAMS PER CORNEA		
Before Incubation	After Incubation	
	Controls	Exposed to Mustard
151.5	86.2	90.4
154.4	89.9	95
148.8	88.1	94.7
151.0		
151.4		
Av.....151.4	88.1	93.3

incubated without injected solution, and that this might have an influence on the formation and disappearance of non-protein nitrogen. At any rate our present conclusions are not affected by this apparent discrepancy.

(4) *Utilization of Serine.* Serine is not only an important constitutent of most proteins; it has been found to function as precursor in the synthesis of phospholipoids, and its chemical constituents suggests a possible relation to carbohydrates, especially amino-sugars. The selection of serine as the first (and so far, the only) amino acid to be tested was prompted in addition by the availability of a specific and sensitive method for serine determination.

Results. Preliminary tests on trichloracetic acid extracts of corneas showed that a substance is present in the cornea which gives the Boyd Logan color reaction and which if calculated as serine amounted to 90 micrograms per cornea. The nature of the substance was not investigated more closely and for convenience it will be designated as F.P. (formaldehyde producing). Determinations in extracts from separated epithelium and stroma demonstrated that the epithelium contains twice as much of this material as does the stroma. The wet weight of the epithelium is not more than 15–20% of that of the whole

TABLE IX

Determination of Endogenous "Serine" (F.P.) in Beef Corneas

Incubated 10 hours at 37°C.
Exposed to Mustard vapor for 15 minutes.
Calculated as Serine in micrograms per cornea.

CONTROLS		EXPOSED TO MUSTARD	
Stroma	Epithelium	Stroma	Epithelium
35.1	69.1	74.6	48.5
23.2	51.9	42.6	32.5
26.2	44.0	44.0	30.3
29.3	64.7	64.0	50.0
33.2	71.7	47.2	47.2
Av.....29.6	60.3	56.5	41.7
Whole cornea 89.9		98.2	

cornea. The concentration of F.P. is, therefore, about 10 times higher in the epithelium than in the stroma.

On incubation of the corneas the total amount of F.P. remains practically unaltered both in normal samples, and after exposure to mustard. In the exposed samples, however, a marked change can be observed in the distribution of F.P. between the tissue components. In the period of incubation which we used, the ratio of concentration of F.P. in epithelium to that in stroma dropped from the normal level of 10:1 to less than 4:1 in corneas which had been exposed to mustard but remained unchanged in incubated control samples (Table IX).

Since the concentration of F.P. in the cornea is relatively low and

remains constant during incubation we concluded that the utilization of serine might be investigated if a relatively large amount of this substance was injected into the tissue. The amount of injected serine used in the following experiments was 10–20 times the amount of endogenous F.P. On incubation of such injected tissues at 37°C. serine was found to disappear in normal corneas at a rate of about 25 micrograms per cornea per hour. After exposure to mustard this rate drops to 7 micrograms per cornea per hour (Table X).

TABLE X

Effect of Mustard upon the Utilization of Serine by Beef Corneas

Amount injected: 2 mg. serine per cornea.
Determinations made on pooled samples of 3 corneas each.
Results in micrograms per cornea.

BEFORE INCUBATION		INCUBATED FOR 10 HRS. AT 37°C.			
		Unexposed		Exposed to Mustard	
Controls	Injected	Controls	Injected	Controls	Injected
88	897	79	673	93	857
93	895	78	690	91	833
	1120	77	957	120	1100
	1097	92	880	93	1107
	1120	84	793		957
	1119		800		980
Av. per Cornea.....91	1041	82	799	99	972
Serine Utilized			242		69

The rate of disappearance of serine was also followed by manometric measurements of amino nitrogen in the extracts. These two sets of measurements yielded the same rate of disappearance of injected serine, and indicated that in the utilization of serine its amino nitrogen is transferred to some non-extractable component of the tissue, while any non-nitrogenous residue is so altered as to be no longer oxidizable to formaldehyde by periodic acid (Table V).

Serine is the first nitrogenous compound, the utilization of which we have found impaired after exposure to mustard. Due to the slow rate of normal utilization of serine by the cornea, we have so far been unable to isolate any of its breakdown products, and no data are available which would suggest the mechanism of its utilization. It is plain,

however, that the serine nitrogen is transferred to some nonextractable tissue component, and that the process involves more than the simple oxidative de-amination of serine, for hydroxypyruvic acid, the product of such a reaction, would give formaldehyde on oxidation with periodic acid and hence be titrated as serine.

We are unable to throw any light on the endogenous substance which yields formaldehyde on oxidation with periodic acid. Exogenous serine is utilized in the cornea at an appreciable rate whereas the endogenous F.P. does not disappear from the tissue on incubation. It may well be that this endogenous material is normally produced as rapidly

TABLE XI

Utilization of Serine as Determined by Amino-Nitrogen Measurements

Amount injected: 2 mg. serine per cornea.
Results in micrograms nitrogen per cornea.

BEFORE INCUBATION		AFTER INCUBATION	
Controls	Injected	Controls	Injected
113.5	322	118	271
109	306	94.8	282
107	312	92.5	272
110	300	84	274
	308	108	258
			269
Average 109.9	309.6	99	271
Injected minus Controls.....	199.7		172.1

Utilized per hour: 2.76 micrograms nitrogen or 21.0 micrograms serine.

as it is consumed and that its level is thus kept constant on incubation. Since the utilization of serine is impaired after exposure to mustard, one might expect endogenous serine to accumulate in the tissue after exposure, but this is not necessarily so because the mustard injury may inhibit the formation of serine as well as its consumption. In any case, the inhibition of utilization of serine does not account for the increased NPN found after exposure to mustard.

SUMMARY AND DISCUSSION

The stability of the extractable non-protein nitrogen content of beef corneas on prolonged incubation was a gratifying finding, since it indi-

cated that the tissue was not undergoing autolysis. Indeed, the capacity of the tissue to utilize ammonia shows that synthetic processes are still going on in the cells. These findings strongly supported our previous conclusions that, with the simple techniques used, the corneas could be maintained alive for many hours in the incubator.

The extractable non-protein nitrogen content of the cornea increases after exposure to mustard. Approximately one half to two thirds of the increased NPN is titratable as amino nitrogen, indicating that the excess NPN is derived from proteins. We have attempted to demonstrate the presence of denatured proteins in the tissue after exposure to mustard, but the available methods proved insufficiently sensitive to give us an answer to this question.

Certain quantitative relations between the amount of mustard taken up by the cornea and the amount of NPN released are of interest. With exposures of 10 minutes we estimate that 6 micrograms of mustard is taken up per square centimeter of cornea, or 24 micrograms per cornea. Approximately one sixth of this, or 4 micrograms, is bound by the tissue. This exposure results in the formation of about 20 micrograms of NPN or, roughly, 70 moles of nitrogen for each mole of mustard bound. This is roughly the order of magnitude of accessible SH groups to nitrogen in some proteins.

The failure of the excess NPN to increase indefinitely with increased dosage of mustard raises interesting problems. It may be supposed that with increased exposure some proteolytic enzymes are inactivated. However, this should result in greater final NPN production with greater exposure to mustard even if the rate of production of the excess NPN were slowed. This we did not find. In a short series of experiments we compared the NPN production after exposure to mustard on aerobic and anaerobic incubation. Under anaerobiosis proteolytic enzymes are generally activated, but the excess NPN produced under these conditions was somewhat less than on aerobic incubation. It would seem possible, therefore, that oxidation of some proteins that had reacted with mustard may enhance their susceptibility to hydrolysis. This suggestion is made in order to show a possible relation between the excess NPN formed on incubation after exposure to mustard and the loosening of the corneal epithelium which occurs after exposure. The dosage range eliciting these two effects is identical. The increased

NPN appears before the loosening of the epithelium. The cohesive boundary can be destroyed by proteolytic enzymes. Anaerobic incubation of mustard exposed corneas protects the tissue against loss of cohesion.

The effect of mustard on serine metabolism revealed many points of similarity to its effect on lactate metabolism. Both metabolites are normally consumed by the intact cornea but not by the denuded stroma, and the consumption of each is inhibited after exposure to mustard. The endogenous formaldehyde producing material which we have titrated as serine is normally present in far higher concentrations in the epithelium than in the stroma. After exposure to mustard the concentration of this material rises in the stroma and falls in the epithelium. A possible explanation of this finding would be in inhibition of the transfer of this substance from stroma to epithelium. We have performed one preliminary experiment to find out whether there is a corresponding active transfer of exogenous serine. After injection of serine into the stroma, and subsequent incubation, the concentration in the epithelium was found to rise rapidly reaching a level far higher than that in the stroma. . The calculated concentration of serine in the epithelium in this experiment remained much higher than that in the stroma even after subtracting the normal titration values of the endogenous formaldehyde producing substance.

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XVI. COMPARISON OF THE EFFECTS OF MUSTARD, ULTRAVIOLET AND X-RADIATION, AND COLCHICINE ON THE CORNEA*

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The analysis of the effects of mustard and the nitrogen mustards reported in the preceding papers cannot fail to raise in the mind of the reader many questions as to the general similarity between the tissue reactions to these agents and those following X-ray and ultraviolet irradiation. To a lesser extent similarities may be noted to the reactions following the administration of colchicine. Equally striking similarities exist between the effects of these agents in producing erythema, vesication and ulceration of the skin. The nature and distribution of the visceral lesions following systemic administration of the mustards is remarkably similar to that produced by exposure to X-ray and other penetrating radiations.

In spite of these similarities some differences in the tissue reactions to these various agents are obvious, and a detailed quantitative analysis on a single tissue would seem desirable. For this purpose the cornea possesses many advantages, both in accessibility to the various agents, and in respect to the availability of quantitative methods for the study of various effects. The data available for such a comparison are, unfortunately, by no means complete. They suffice, however, to indicate some specific differences between the effects of several of the agents.

The cytological studies in regard to mitotic and nuclear changes were performed mainly on rats but have been amplified for the present purposes by studies on beef corneas. There is a general similarity in the reaction of the two species, but some minor differences will be noted below. Quantitative studies on the loosening of the corneal epithelium were performed on beef corneas. Less exact methods applied to the study of this phenomenon on rats' corneas revealed no differences. The analysis of metabolic effects was made exclusively on

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beef corneas. In addition to the data on the metabolic effects of mustard on the cornea presented in the preceding papers, a study of the non-protein nitrogen content of the tissue after exposure to ultraviolet light and to colchicine is reported below.

AGENTS TO BE COMPARED

1) *Sulfur and Nitrogen Mustards.* Studies on the altered metabolism of the cornea after exposure, reported in the preceding papers, have been limited entirely to sulfur mustard, and no data are available concerning the effects of the nitrogen mustards on corneal metabolism. Studies on mitotic and nuclear changes and on loosening of the epithelium revealed no differences between mustard and three of the nitrogen mustards.

Estimates of the amount of mustard absorbed by the tissue in the production of a given effect must be given with considerable reserve. In respect to those doses which produce the metabolic disturbances and the epithelial loosening in the beef cornea, we are on reasonably solid ground, having measured directly the mustard uptake of the beef cornea under the conditions of exposure that we were using. In order to reach an estimate of the dose required for mitosis inhibition two extrapolations were necessary. In the first place, we have assumed that the uptake of mustard per square centimeter in the rat's cornea under similar conditions of exposure is the same as in the beef, and have then measured the time of exposure to mustard vapor required to produce a lesion of given clinical severity in the rat. We have then reproduced lesions of the same clinical severity by administering to the eye a drop of mustard dissolved in hexane. Using instillation of much more dilute solutions of mustard in hexane we have explored the effect on inhibition of mitosis. The estimate we have given for the dosage related to different degrees of mitosis inhibition rests on the assumption that the amount of mustard absorbed from a hexane solution, or from vapor, are the same if they cause lesions of the same severity; and that the amount absorbed from increasingly dilute solutions of hexane is proportional to the concentration. Comparison of rat and beef corneas exposed to the same doses of mustard vapor indicates that the rat's cornea is about twice as susceptible to this agent in respect to loosening of the epithelium as the beef.

2) *Ultraviolet Light.* A report of many of our findings on the effect

of ultraviolet light on the rat's cornea has been published elsewhere (1). Some additional data relating to the effects of specific wave lengths have been obtained in collaboration with Dr. Kinsey at the Howe Laboratory and later with Dr. Hollaender at the National Institute of Health and will be included in brief summary below.

A comparison of the effects of ultraviolet light on beef and rat corneas in producing epithelial loosening and nuclear fragmentation revealed that in order to elicit comparable reactions, a dose was required for beef tissue 10–20 times that required for the rat. Moreover, nuclear fragmentation develops much more slowly in the beef than in the rat after both ultraviolet and mustard exposures. In the rat's corneal epithelium, clumping of the nuclear chromatin is visible 2–3 hours after exposure, and disappearance of the nuclear membrane with dispersal of the chromatin into the cytoplasm is abundantly shown after 6 hours of incubation. In the beef, clumping of the nuclear chromatin is visible after 6 hours at 37°C., but in most of the affected cells the nuclear membrane remains intact up to 18 or 20 hours after exposure. The chromatin in the affected cells in the beef usually forms a solid mass in the center of the nucleus surrounded by an eosinophilic zone, or, alternatively, it is adherent to the nuclear membrane. Similar patterns are seen occasionally in the rat's corneal epithelial cells. Unless otherwise specified, the dosage of ultraviolet was controlled by varying the time and distance of exposure, using a cold quartz mercury arc as the source. At the standard distance of 5 cms. this source yielded approximately 10^6 ergs/cm^2 per minute.

3) *X-ray, Grenz ray, Beta ray.* A comparison of the effect of hard (200 KV) and soft (24 KV) X-ray was made in collaboration with Dr. Gustav Bucky. For equal exposure in terms of roentgen units (ionization produced per cm^3) equal effects were produced. Beta ray from radon contained in a thin glass capsule was applied with the capsule 5 mm. from the apex of the cornea. The results were essentially similar to those with X-rays. Under the conditions of our experiments, exposure to Beta radiation equal to 1 gram second (radium) gave an inhibition of mitosis equal to that of approximately 50 roentgen units. Owing to the geometrical relations between the applicator and the cornea, the exposure to Beta rays at the periphery of the cornea was slightly less intense than at the center, and recovery from the inhibition

began sooner at the periphery. Full report of our experiments with these ionizing radiations will be given elsewhere.

4) *Colchicine.* Results on mitotic activity in the corneas of rats following systemic administration of colchicine have already been reported (2). The results of intracorneal injections are given below.

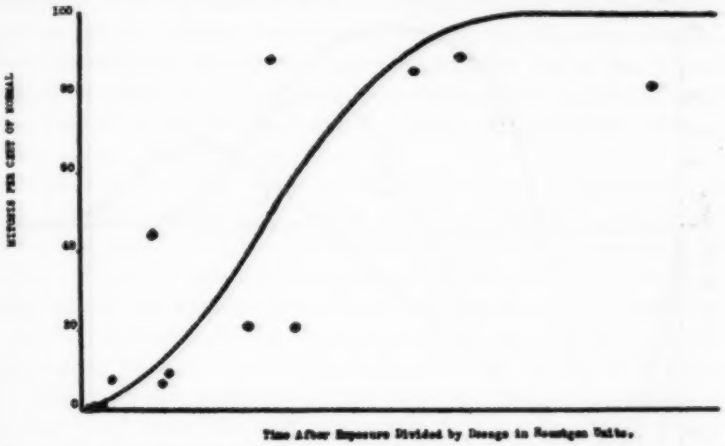

FIG. 1. MITOSIS INHIBITION AND RECOVERY FOLLOWING EXPOSURE TO X-RAYS
The curve is a composite of results obtained with doses of X-rays varying from 50 to 2000 r units. The curve is plotted with mitosis percent of normal as ordinate versus time after exposure divided by dosage in r units as abscissa. Each dot represents the average of counts of 2–4 animals.

EFFECTS

I. *Mitosis Inhibition.* With X-ray over a range of exposures from 50 to 2000 roentgen units, inhibition of mitosis was immediate and complete. Similar results were obtained with Beta rays. Recovery of mitotic activity followed an S shaped (autocatalytic) curve as shown in Fig. 1. With increasing doses, the slope of the recovery curves was flatter, and the time to 50% recovery was proportional to the dose. Since mitotic activity was initially completely inhibited with doses that produced varying inhibitions of the recovery mechanism, it seems possible that X-rays injure two components of the mitotic mechanism: (A) a component immediately necessary for mitosis and (B) a

component required to recover from the inhibition. (B) may, for instance, be required to form (A).

With ultraviolet and mustards, the inhibition of mitosis developed slowly over the course of some hours, the speed with which the inhibition developed being roughly proportional to the dose (Fig. 2). Recovery from the inhibition followed curves similar to those obtained

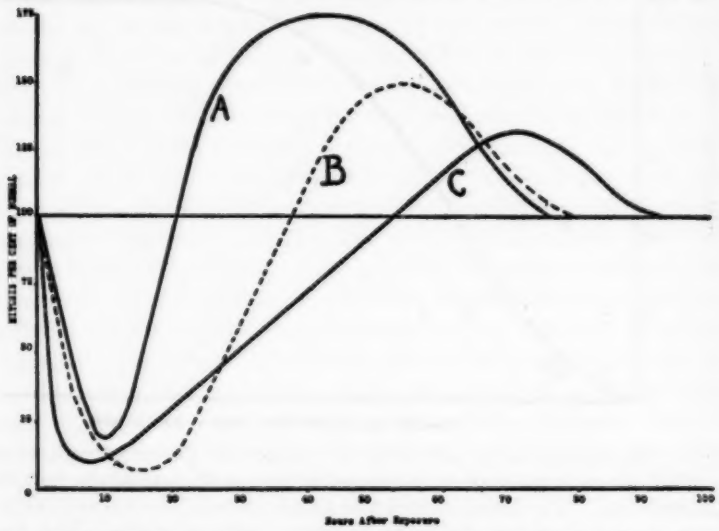

FIG. 2. MITOSIS INHIBITION AND RECOVERY FOLLOWING EXPOSURE TO TWO DIFFERENT DOSES OF ULTRAVIOLET (A AND C) AND TO A SINGLE DOSE OF NITROGEN MUSTARD (B)

The dose of Ultraviolet producing the results shown in Curve C is double that or Curve A. Curve B is the same as that shown in a previous report (4).

with X-rays. Comparing inhibitions of equal duration produced by ultraviolet and mustard on the one hand with those produced by X-rays on the other, it was found that with mild exposure (6–12 hours inhibition) the inhibition was less complete with ultraviolet and mustard than with X-ray.

If the supposition about a possible dual injury with X-ray is correct, then ultraviolet and mustard do not injure the (A) component but may injure the (B) component similarly to X-rays. The matter, however,

is more complicated. Over the range of dosage we have studied, the inhibitions produced by ultraviolet and mustard are quite regularly followed by a period of mitotic activity exceeding that of the control, while we have observed no such recovery overshooting following X-radiation. On the other hand, recovery overshooting has been observed following mitosis inhibition from X-radiation on grasshopper neuroblast (3). Perhaps we have not yet reached sufficiently low doses of X-radiation to demonstrate recovery overshooting in the cornea, but in any case inhibitions of similar durations are associated with recovery overshooting in the case of mustard and ultraviolet, and are without this in X-radiation.

Several alternatives present themselves as possible explanations of the recovery overshooting. Some normally inhibitory mechanism may be removed, or some necessary metabolite may accumulate in excess. This matter has already been discussed in a previous paper (4) where evidence was presented indicating that during the overshooting following mustard there was no disproportionate excess of cells in the premitotic state. Similar results have been obtained on the overshooting following ultraviolet. The recovery overshooting following mustard and that following ultraviolet radiation was tested with adrenalin, and it was found that the mitotic activity was just as susceptible to inhibition by adrenalin during the overshooting as in normal controls.

II. *Nuclear Fragmentation.* The phenomena of nuclear fragmentation following exposure to mustard (5) and to ultraviolet (1) have been reported in previous papers. In respect to the cytological pattern of development, no differences were noted between the two agents when studies were made on the same species. The incubation periods and the time of development through various stages were the same. In both cases the development of nuclear fragmentation was inhibited by anoxia and by reduced temperature of incubation. In each case evidence has been adduced suggesting that the nuclear fragmentation represents, perhaps, a pathological and distorted form of mitosis.

In spite of the considerable similarities between nuclear fragmentation produced by mustard and by ultraviolet, important differences are still present. Under normal conditions only a few cells, and these only in the basal layers of the corneal epithelium, are susceptible to nuclear

fragmentation by mustard and nitrogen mustard. We have suggested that these susceptible cells may be those which are in a premitotic state (5). A variety of conditions, e.g. previous exposure to adrenalin, to hypotonic, and to acid solutions, greatly increase the number of susceptible cells, and under these conditions cells in the superficial, normally non-mitosing layers may also show nuclear fragmentation after exposure to the mustards.

In contrast to these findings ultraviolet produces fragmentation mainly in the superficial layer of corneal cells, and, in the rat, cells showing this reaction are regularly abundant, irrespective of the conditions of pretreatment so far tested. It should be noted that mitotic activity is normally absent in this cell layer. It might be supposed that exposure to ultraviolet entails an effect sensitizing cells to nuclear fragmentation similar to that of the various agents which were shown to be sensitizing when followed by mustard. We are forced to the conclusion that while the pathological processes of nuclear fragmentation produced by ultraviolet and by mustard are apparently identical, the state of cells responding in this way to the injury is different for the two agents, and hence that the primary injuries by these two agents are different.

In the corneal epithelial cells we have seen no similar nuclear fragmentation following X-ray. We have not, so far, tested whether the pretreatments which sensitize the corneal cells to nuclear fragmentation by mustard have a similar effect in relation to X-ray. It may be noted that nuclear fragmentation in the corneal epithelium is associated with a marked increase in the amount of Feulgen positive material similar to that seen in normal mitosis. Hevesy (6) has recently reported that in certain tissues which he has studied, mitosis inhibition produced by X-ray is associated with an inhibition in the synthesis of desoxyribose-nucleic acid. An inhibition of desoxyribose-nucleic acid synthesis might make nuclear fragmentation impossible since in the cornea this phenomenon is associated with the accumulation of Feulgen positive material. It may be noted, however, that nuclear fragmentation occurs in the bone marrow and in lymphoid tissue after X-radiation. No information is available as to whether, in the tissues, nuclear fragmentation is also associated with the synthesis of Feulgen positive material.

It may be pointed out that after exposure to X-ray and Beta ray pathological mitoses in the form of unequal separation of the chromosomes at anaphase, or multipolar mitotic spindles are occasionally seen. Phenomena of this type have been frequently described in relation to the effects of ionizing radiations on other tissues. They appear to us to have no relation to what we have defined as nuclear fragmentation, and we have not seen mitoses of this type following mustard or ultraviolet exposures.

Further evidence that mitosis inhibition and nuclear fragmentation result from injuries that are at least to some extent independent is seen in Table I. Taking the dose which produces 24 hours inhibition of mitosis as the basis of comparisons, nuclear fragmentation is produced by one third this dose in the case of ultraviolet, ten times this dose in the case of mustard, and not with two hundred times this dose in the case of X-ray.

Colchicine does not inhibit the onset of mitosis in the corneal epithelium in the doses that we have studied, but stops the process in metaphase without spindle formation. After prolonged arrest in metaphase the chromatin tends to clump irregularly and eventually fragments become dispersed in the cell. The final result is similar to that produced by mustard and ultraviolet. Since only spontaneously mitosing cells reach this state under colchicine the lesion is confined to the basal epithelium. The time required for a cell in arrested metaphase to undergo this form of chromatin dispersal is markedly influenced by the temperature. If rats' eyes are removed 40 minutes after systemic colchicine injection (50 mg. per Kg. bodyweight) and incubated in moist chambers, the chromatin dispersal occurs in 4 hours at 38°C., in 8 hours at 30°C., and it only begins to appear between 20 and 24 hours at 25°C.[1]

III. *Loosening of the Corneal Epithelium.* Ionizing radiations, ultraviolet, mustard, and colchicine, all produce loosening of the corneal epithelium, but at doses that vary widely relative to the mitosis inhibiting dose. The two effects appear, therefore, to be independent. The question of the possible relation between nuclear fragmentation

[1] It is of some interest in this connection that the systemic toxic effects of colchicine have been reported to be markedly dependent on temperature in hibernating (7), and in cold blooded animals (8).

and epithelial loosening in the case of mustard has already been discussed (4). Both processes are suppressed by anoxia and hence both require oxygen for their development, but the temperature coefficient of nuclear fragmentation is steeper ($Q_{10} = 3.5$) than that for loosening of the epithelium ($Q_{10} = 3$), consequently either phenomenon can be made to precede the other depending on the temperature of incubation. Moreover, under basal conditions (absence of preceding adrenalinemia) in vivo, nuclear fragmentation is minimal but the loosening process is not diminished.

We believe, therefore, that the epithelial loosening process is independent of nuclear fragmentation in spite of the fact that there are some apparent correlations between the two processes. For instance,

TABLE I

Comparison of Dosage Level with Different Agents in Rats' Corneas

	MUSTARD MICROGRAM/CM²	ULTRAVIOLET EXPOSURE TIME, MINUTES	X-RAY ROENTGEN UNITS
24 hours Mitosis Inhibition	0.01	1½	100
Threshold Nuclear Fragmentation	0.1	½	
Loosening of the Epithelium	1.0	3	20,000*

* The loosening of the corneal epithelium occurs as part of the late ulcerative reaction and must be only very indirectly related to the initial ionizing effect of the radiation.

ultraviolet, which causes the most abundant nuclear fragmentation, produces epithelial loosening at relatively low doses, while X-ray which fails to cause nuclear fragmentation, requires the highest dose to produce epithelial loosening when the dose for mitosis inhibition is taken as the standard of reference (Table I).

Colchicine administered systemically in doses fatal to many of the rats in 18–24 hours (2 mg./kilo) fails to produce loosening of the corneal epithelium. Intracorneal injection of as little as 0.1 micrograms in beef corneas produced definite loosening of the epithelium (Table II). Since the wet weight of the beef cornea is about one gram this amounts to a concentration of 0.1 milligrams per kilo. It follows that either the beef cornea is much more susceptible to the loosening effect of colchicine than the rat's, or that systemically injected colchicine in the rat

does not achieve a uniform distribution throughout the animal.[2] Since some colchicine arrested mitoses appear in the beef cornea with injections of 1 microgram of colchicine into the isolated tissue, the loosening of the epithelium by this agent has no direct relation to inhibition of mitosis.

IV. *Increased Non-Protein Nitrogen.* In the preceding paper (9) experiments were reported showing that after exposure to mustard there is an increase in extractable NPN in the corneas. Using the

TABLE II

Effect of Colchicine upon the Adhesion of the Corneal Epithelium

Dose: injected in 0.25 ml. of 0.3% saline.
Incubation: 10 hours at 37°C.—A; 10 hours at 28°C.—B.

AMOUNT INJECTED	A*	B*
Controls	−−−−−±−	−±
0.1 micrograms	−++	+±
1.0 micrograms	+++	+±
10.0 micrograms	++++++++++	++
100.0 micrograms	+++	+±
500.0 micrograms	+++	±±
1.0 micrograms	+++	±+

* Each symbol in these columns represents an experiment on a beef cornea.
The epithelial adhesion was tested with a scraping weight of 90 gms.
− indicates less than ½ of epithelium removed.
± indicates about ½ of epithelium removed.
+ indicates more than ½ of epithelium removed.

same techniques, a series of experiments were performed comparing the effect of mustard with that of ultraviolet light and of colchicine. After exposures of ultraviolet light capable of producing loosening of the epithelium, an increased NPN content was found similar to that following similar exposures to mustard (Table III). The time of development, the amount of increase, the proportion of amino nitrogen in the NPN, and the temperature dependence of the effect was similar with these two agents. These results support our previous conclusion

[2] The colchicine preparation which we used was the usual commercial one. The epithelial loosening which we observed following injection of this material may be due to some impurity present and not to the colchicine itself.

that the increased NPN is not derived from cells undergoing nuclear fragmentation for such cells are much more abundant after ultraviolet than usually the case after mustard.

No change in the extractable NPN content of the cornea was noted after injections of colchicine (Table IV). If the increased NPN after

TABLE III

Effect of Ultraviolet Irradiation upon the Non-Protein-Nitrogen Content of Beef Corneas[5]

Treatment: irradiated for 30 min. at a distance of 2cm. from source.
Incubation: 6 hours at 37.5°C.

MICROGRAMS NPN PER CORNEA	
Controls	Treated
247	273
239	283
252	297.5
253.5	274
259.5	321
267	336.5
257.5	302.5
251.5	282
275.5	296.5
219.5	313
216.5	258.5
245	299.5
244.5	229.5
232.5	276.5
269.5	246
291	275
261.5	267.5
277.5	263
Average.....249	277

Average increase per cornea = 28 micrograms NPN.

mustard is connected with the loosening of the corneal epithelium, then the mechanism of loosening of the epithelium by colchicine must be different from that by mustard. Since nothing is known concerning the mechanism of action of colchicine in chemical or metabolic terms, no interpretation of this negative finding can be offered at the present. A comparison of the effects of mustard, ultraviolet, and colchicine on the beef corneas is shown in Table V.

TABLE IV

Effect of Colchicine upon the Non-Protein-Nitrogen Content of Beef Corneas

Amount injected: 10 micrograms colchicine in 0.25 ml. of 0.3% saline.
Incubation: 10 hours at 37°C.

MICROGRAMS NPN PER CORNEA	
Controls	Treated
278	290
295.5	283.5
201	286
229.5	247
238	232
248	224.5
238.5	243
246	241.5
276.5	228.5
271.5	242
263.5	247
	276.5
Average.....253	253.5

TABLE V

Relation between Proteolysis, Epithelial Loosening, and Nuclear Fragmentation in Beef Corneas

TREATMENT	CONDITIONS OF INCUBATION	PROTEOLYSIS (increase in NPN)		LOOSENING OF EPITHELIUM*		NUCLEAR FRAGMENTATION IN EPITHELIUM†	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1. Exposed to Mustard Vapor: 15 min. Uptake 0.6 micrograms per cm.² per minute	10 hours at 28°	+	+	+	−	−	−
	20 hours at 29°	+	+	+	−	±	−
	10 hours at 37°	+	+	+	−	±	−
2. Irradiated at 2 cm. distance from ultraviolet source: 30 min.	6 hours at 37°C.	+		−	−	± ‡	−
	12 hours at 30°C.	−		−	−	∓ ‡	−
	20–24 hours at 30°C.	+		+	−	− ‡	−
3. Injected with 10 micrograms Colchicine	10 hours at 37°C.	−		+	−	±	−
	10 hours at 28°C.	−		±	−	−	

* The adhesion of the epithelium was tested with a scraping weight of 90 gms. Significance of symbols same as in Table II.

† − indicates no nuclear fragmentation.
± indicates intranuclear chromatin clumping.
+ indicates loss of nuclear membrane and dispersal of chromatin fragments.

‡ Superficial layers.

SUMMARY

Some of the tissue reactions to X-ray, ultraviolet radiations, and colchicine, have been compared with similar reactions to mustard. Among the agents compared, the effect of ultraviolet resemble those of mustard most closely, though with definite quantitative differences and with differences in relative susceptibility among various layers of corneal epithelial cells. If the mitosis inhibition produced by X-ray has any element in common with that produced by ultraviolet and mustard, then the effects of both types of agents are complex, each involving some factors not shared by the other. In the case of ultraviolet and mustard there may be some connection between the factors which lead to mitosis inhibition and those which lead to nuclear fragmentation, but nuclear fragmentation was not found in the corneal cells after X-ray in spite of marked inhibition of mitosis. Neither mitosis inhibition nor nuclear fragmentation appears to be directly connected with the loosening of the corneal epithelium nor with the increased NPN content of the cornea found after ultraviolet or mustard exposure. If the increased NPN following mustard is connected with the mechanism producing loosening of the epithelium which occurs with the same intensities of exposure, then the mechanism of loosening the epithelium by colchicine is different from that induced by mustard.

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XVII. SUMMARY AND SOME POSSIBLE INTERPRETATIONS

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A general summary and discussion of the effects of mustard on nuclear activities in the corneal tissues is given in the preceding paper. The present paper concerns the effects of mustard on corneal metabolism and the possible relation of these effects to the loosening of the corneal epithelium.

I. *Metabolic Interaction between the Corneal Epithelium and Stroma.* The evidence presented in the preceding papers reveals that the carbohydrate metabolism of the corneal epithelium is similar to that found in many other tissues. It has a cyanid sensitive oxygen uptake and contains cytochrome oxidase. It consumes glucose, glycogen, lactate, and pyruvate. Under aerobic conditions and adequate supply of carbohydrate, the oxygen uptake equals that required for complete combustion of the carbohydrate consumed. Under anaerobic conditions, lactate is produced equivalent to the loss of glucose and glycogen. Glycolysis is inhibited by fluoride and iodoacetate, and under these circumstances phosphate esters accumulate, indicating that the glycolytic system follows the usual pathways. In the absence of adequate carbohydrates, the oxygen uptake remains normal or rises. This is not associated with any increased non-protein nitrogen. Consequently, the alternative metabolites are probably fats.

In contrast with the conventional metabolic pattern of the epithelium, the stroma has no oxygen uptake and can utilize neither lactate nor pyruvate. Nevertheless, it is capable of utilizing glucose at a rate per cell about twice that of the epithelium. Lactate is produced by the stroma but cannot be consumed by it. In the isolated cornea the lactate produced by the stroma is utilized by the epithelium, and constitutes an appreciable fraction (perhaps about 25%) of the total carbohydrate supply of the epithelium. Moreover, the aerobic glucose consumption of the stroma with the epithelium in place appears to be considerably less than in the freshly denuded stroma, indicating a control of glucolysis in the stroma by the oxidative metabolism of the adjacent epithelium, analogous to the familiar intracellular Pasteur effect.

The denuded corneal stroma, however, rapidly loses its capacity to utilize glucose, and an examination of the distribution of phosphate esters in the tissue suggests that the failure in glucose consumption is due to an exhaustion of the capacity of the tissue to transfer phosphate from glycerophosphate to hexose. We have concluded, therefore, that the epithelium normally assists the stroma in maintaining a supply of carriers for energy-rich phosphate transfers.

After exposure of the cornea to mustard in appropriate dosage, the consumption of stroma lactate by the epithelium is markedly inhibited. This inhibition occurs even when the epithelium is still able to consume its endogenous stores of lactate at an almost normal rate. Consequently, the epithelium must possess a special mechanism for the utilization or transfer of the stroma lactate. In either case it is reasonable to suppose that this mechanism is located at, or close to the stroma-epithelium boundary. Failure in the utilization of stroma lactate after exposure to mustard is associated with a corresponding decrease in O_2 uptake, indicating that the metabolites (fats) which normally serve as alternates when the supply of lactate is reduced, cannot be consumed after mustard injury. If the same injury is responsible for inhibition of the utilization both of lactate and of its alternates, a link in the hydrogen transport system common to both of these metabolites must be damaged by mustard. This leads to the conclusion that a special hydrogen transport system is present for the utilization of the stroma lactate, independent of that concerned with the utilization of endogenous lactate in the epithelium.

II. *Transfer of Metabolites from Stroma to Epithelium.* Lactate, pyruvate, serine, and probably butyrate, are not consumed by the denuded stroma but are consumed by the cornea with the epithelium intact. The question, therefore, arises as to whether their penetration into the epithelium is by simple diffusion or by active transfer.

(1) Pyruvate. Cogan and his co-workers (1) have shown that the stroma-epithelium boundary is highly impermeable to ions, and his conclusions are strongly supported by our own measurement of the electrical resistance of the tissue (2). When sufficient pyruvate is placed in the stroma, the epithelium consumes 400 micrograms of pyruvate per hour. The movement of anions at this rate is equivalent to an electrical current of 0.1 milliamperes. The electrical resistance of the boundary is 500,000–1,000,000 ohms per square millimeter or

1,000 to 2,000 ohms per cornea. Consequently, energy equivalent to 0.1–0.2 volt would be required to transfer the pyruvate through the resistant boundary. The required energy seems excessive in relation to the diffusion potential of a 0.01 molar solution of pyruvate in the presence of physiologic concentrations of electrolytes.

As a matter of fact, Miles (3) has found a potential of approximately 0.001 volt associated with the corneal surface, but of the opposite sign to that required for the movement of anions from stroma to epithelium. Whether this potential arises in the cornea or in some of the intraocular tissues has not been decided. There is, furthermore, a difference in redox potential between epithelium and stroma, but if there is any redox interaction between epithelium and stroma in the cornea similar to that which has been found in some other tissues (4, 5), the compensating ionic exchange would be in the opposite direction to that required to transfer anions from stroma to epithelium.

In view of these difficulties we are compelled to consider the possibility that pyruvate may be converted into an uncharged molecular species in preparation for its transfer to the epithelium. One possibility is that the pyruvate might be esterified onto some component of the boundary layer, and de-esterified on the opposite side. A second possibility is that pyruvate which, as is well known, forms unusually stable condensation products, may be transferred to the epithelium in the form of an unionized polymer. Since the transfer and utilization of pyruvate remains unaffected after mustard, the precise mechanism of pyruvate transfer need not be pursued further.

(2) Lactate. The evidence against simple diffusion as the mechanism of transfer is considerably stronger in respect to lactate than pyruvate. The amount of stroma lactate consumed by the epithelium is only 25 micrograms per hour as compared with 400 micrograms pyruvate per hour, but the concentration of lactate in the epithelium except under very special experimental conditions is either equal to or higher than that in the stroma. In corneas freshly removed from the animals, the concentration of lactate in the epithelium is twice that in the stroma, and this difference can be maintained unchanged for many hours of incubation if glucose is supplied. If simple diffusion between the two tissues were possible equalization of the lactate content would be expected. Furthermore, the failure of

the stroma lactate level to rise significantly under these conditions indicates either that lactate is being transferred against an adverse concentration difference, or that the stroma has no glucose consumption. The latter alternative is contradicted by direct experimental measurements on the isolated stroma.

When excess lactate is injected into the stroma it is consumed at the rate of 25 micrograms per hour irrespective of its concentration in the stroma. This is, therefore, the upper limit of the activity of either the transfer or the consuming mechanism.

In spite of the strong evidence against transfer of lactate by diffusion, we have, so far, failed to obtain correspondingly direct positive evidence for active transfer. We have sought for conditions under which we could demonstrate a decline in stroma lactate during a period in which the concentration of lactate in the epithelium is higher than that in the stroma. Unfortunately, in fresh tissue at the time when the concentration difference is found, the cornea contains an appreciable reserve of glucose most of which is in the stroma. Until these reserves have been used up no decline in stroma lactate concentration is to be expected, since production of lactate in the stroma should balance its losses by transfer to the epithelium. By the time that the glucose reserves have been exhausted, and the stroma lactate level has begun to fall, the epithelium has also exhausted its quota of glucose and begun to consume its endogenous lactate. Consequently the concentration difference required to prove active transfer has disappeared. Moreover, in studying such a sequence of events, different corneas have to be sampled after different periods of incubation. Since individual variations in the lactate and glucose stores are considerable, rather large differences in the experimental results are required to be significant.

Indirect evidence of active transfer was, however, obtained through the demonstration that the transfer of stroma lactate, as well as its utilization, is inhibited by mustard. This is shown in experiments previously reported (6) in which, after exposure to mustard, the corneas were incubated anaerobically for several hours at room temperature. By this procedure the effects of the mustard injury are given time to develop while the lactate reserves in the tissue are maintained at a normal level. Subsequent aerobic incubation at 29°C enabled the

epithelium to consume its endogenous lactate and to produce a concentration ratio between the two tissues favorable to diffusion from stroma to epithelium. Nevertheless, the stroma concentration of lactate was fairly well maintained on many hours of incubation.

The evidence that mustard inhibits both the transfer and utilization of stroma lactate suggests that the two mechanisms involved may be closely linked. We have already suggested that the mechanism for utilizing the stroma lactate is at or near the stroma-epithelium boundary. If we assume that lactate is oxidized at the boundary and converted to pyruvate then the simultaneous inhibition of both the transfer and the utilization of stroma lactate by a single mustard injury would be explained. Similar oxidation of extracellular substrates on the stroma side of the stroma-epithelium boundary have been shown to take place in the ciliary body (4) and choroid plexus (5). In these organs the oxidative interaction across the boundary is associated with a secretory transfer of water and electrolytes. Such a transfer, however, depends on the nature of the boundary. No evidence of such a secretory transfer in the cornea is available, though Cogan's (7) finding that the corneal stroma is maintained in a state of detergescence when the epithelium is present, and swells by uptake of water in the absence of the epithelium, suggests that the interrelations of stroma and epithelium include some mechanism for the extraction of water from the stroma.

While the hypothesis suggested above regarding the mechanisms of utilization of stroma lactate appears to satisfy practically all the available data, there is one set of experiments not easily reconciled with this hypothesis. When iodoacetate is injected into the cornea, glycolysis is inhibited and the lactate reserves of the tissue are consumed in the presence of iodoacetate, even if a large excess of glucose is present. Mustard inhibits the consumption of the stroma lactate. If iodoacetate is injected into mustard poisoned tissues, the inhibition of lactate consumption is partially reversed. This apparent reactivation of an inhibited system by a second poison constitutes a contradiction of some of the assumptions we have made.

A way out of this dilemma is the following: we have assumed so far that glucose is the only possible source of stroma lactate, and that when the reserves of glucose are exhausted no further lactate production takes place in the stroma. Consequently, we have assumed

when the lactate level in the stroma remained stationary after exposure to mustard that the utilization of stroma lactate was completely inhibited. It may well be, however, that the stroma is able to produce lactate from endogenous stores other than glucose,—for instance glycogen or mucoid,—and that the inhibition observed after exposure to mustard would then represent a balance between the partially inhibited lactate consumption mechanism and the reduced lactate production in the absence of glucose. If this residual lactate production is now inhibited by iodoacetate, a resumption of the decline in stroma lactate would occur, producing an apparent reversal of the mustard inhibition. If this is correct, then the normal rate of utilization of stroma lactate is slightly greater than we have estimated.

(3) Serine. Unfortunately we have but a single experiment directed toward a study of the possible transfer of serine from the stroma to the epithelium, and though this experiment yielded evidence of a strong accumulation of serine in the epithelium further confirmation is required.

The utilization of serine is inhibited by the same dose of mustard required to inhibit the utilization and transfer of lactate. If the mechanism for the utilization and transfer of lactate outlined above is correct, no special difficulties would be encountered in accounting similarly for the utilization and transfer of serine. Both the imine derived from the oxidation of serine and the deaminated product of this imine,—hydroxypyruvic acid,—would be titrated as serine in the analytic procedure used.

(4) Other substances. Butyrate injected into the stroma of the intact cornea is consumed at a rate similar to that for lactate and serine. The consumption of butyrate is inhibited by the same dose of mustard that inhibits utilization of lactate and of serine. We have no data on the possible utilization of butyrate by the denuded stroma, but, since oxidation would appear necessary for its utilization, consumption by the stroma would seem unlikely. If corneas are incubated without added substrate until the reserves of lactate are used up, alternate substrates are burned, and since there is no increase in nonprotein nitrogen under these circumstances the alternate substrate are presumably fats. Consequently, the metabolic systems in the tissue which normally consume lactate can utilize fats as alternates. After exposure to mustard sufficient to inhibit the utilization of lactate, the

oxygen uptake drops in proportion to the reduced carbohydrate consumption. The alternates in this case cannot be utilized. It would appear, therefore, that butyrate might be a suitable example of an alternate to lactate and that its utilization is also carried out by the same mechanism.

Two substances of special interest, glutathione (8) and ascorbic acid (9) have been found present in the corneal epithelium in far higher concentration that in the stroma. No data are at present available to indicate whether these substances reach the epithelium from the stroma, from the tears, or are synthesized locally. Moreover, the existence of reversibly bound acorbic acid has been demonstrated (10); consequently the high concentration of this substance in the epithelium need not indicate an inequality in free ascorbic acid between epithelium and stroma. Non-protein nitrogen is also found in higher concentrations in the epithelium than in the stroma.

III. *Loosening of the Epithelium.* The discussion in the preceding sections yields a picture of the boundary between stroma and epithelium as the seat of complex and intense metabolic activities. Some aspects of this metabolic activity are inhibited by mustard. The same dose of mustard which produces these metabolic effects also causes a loosening of the tissue cohesion at the stroma-epithelium boundary. We must now inquire whether any connections can be indicated between the metabolic phenomena and the pathological event of loss of cohesion.

In the studies on the loosening of the epithelium (11) it was found that proteolytic enzymes on the one hand, and unionized surface active substances, such as butyl and amyl alcohol, on the other, disrupt the coherent boundary. These findings were taken to indicate that the cohesive boundary consists in part of a protein-lipoid multilayer. We can now suggest that the lipoid component of this multilayer is responsible for the relative impermeability of the boundary to ions, and its consequent high electrical resistance.

EFFECT OF IODOACETATE AND FLUORIDE

The tissue cohesion is well maintained in the absence of oxygen and in the presence of cyanide and azide, but loosening of the epithelium occurs after application of iodoacetate and of fluoride. In view of this we have previously suggested that energy from glycolysis may be re-

quired for the maintenance of the cohesive system, and this is supported by the observation that partial loosening occurs after very prolonged incubation (24 hours) in the absence of added substrate, under which circumstances the available substrates for glycolytic break-down are largely exhausted. However, the matter is somewhat more complicated. Iodoacetate produces a very marked inhibition of glycolysis, and causes a loosening of the epithelium. The effect of iodoacetate might, therefore, be the result of an inhibition of glycolysis. With fluoride, which also inhibits glycolysis, a paradoxical situation arises. If fluoride is administered to the cornea in external bathing fluid, it causes a loosening of the epithelium if the tissue is incubated aerobically, but fails to do so under anaerobic incubation. However, when fluoride is injected into the stroma it causes a loosening of the epithelium both in the presence and in the absence of oxygen. Now when fluoride is administered in the external bathing fluid and the tissue is incubated anaerobically, a marked inhibition of glycolysis is observed. Under these experimental conditions the endogenous supply of glucose is rapidly exhausted in the controls without fluoride. Therefore, most of the excess lactate produced in the controls is derived from glycogen in the epithelium. It follows that fluoride supplied externally under anaerobic conditions is able to inhibit glycolysis in the epithelium, and, hence, that glycolysis, at least in the epithelium, is not necessary for the maintenance of the tissue cohesion. Consequently the loosening of the epithelium caused by iodoacetate cannot be attributed to inhibition of glycolysis, at least so far as the epithelium is concerned. The mechanism of loosening by iodoacetate is, therefore, independent of that by fluoride. Iodoacetate does not inhibit the utilization of stroma lactate. It differs from mustard both in this respect and also in the fact that the loosening is produced by iodoacetate both aerobically and anaerobically.

When fluoride is administered and the tissue incubated aerobically, it causes inhibition of glycolysis and also inhibition of utilization of stroma lactate, and the epithelium is loosened. It might be supposed that the failure in utilization of lactate under these circumstances is due to an inhibition of pyruvate oxidation displacing to the left the reaction:

$$\text{Lactate} \leftrightarrows \text{Pyruvate} + 2\text{H}$$

However, the tissue can dispose of pyruvate by dismutation at a very rapid rate even in the presence of fluoride. Consequently, the effects of fluoride on lactate utilization must be on the mechanism that oxidizes lactate, unrelated to any effects that it may have on pyruvate oxidation in addition. We have here a parallelism between mustard and fluoride, both of which inhibit the utilization of stroma lactate and cause loosening of the epithelium. There is, however, an important difference. The loosening by mustard does not occur on anaerobic incubation, that by fluoride injected into the stroma does. Consequently the injury by fluoride is more directly disruptive of the cohesive boundary than that of mustard.

One further conclusion is to be drawn from the fluoride experiments. Under anaerobic conditions fluoride introduced into the stroma causes loosening of the epithelium, but when introduced into the epithelium it is without effect, and therefore, the stroma-epithelium boundary is impermeable to fluoride under anaerobic conditions. When the same experiment is performed aerobically loosening occurs no matter on which side of the boundary the fluoride is presented. It follows either that fluoride can loosen the epithelium by either of two independent mechanisms, or that the boundary between epithelium and stroma is less impermeable under aerobic than under anaerobic conditions. These comments suffice to show that the cohesive system is a highly complex organization, and has several points of vulnerability.

EFFECT OF MUSTARD

In spite of these obvious complexities we can, at least, hold fast to what little we do know about the effects of mustard, and inquire whether there is any possible connection between them. Mustard causes loosening of the tissue on aerobic but not on anaerobic incubation. Consequently, oxygen is necessary for the effect, and either some new oxidative channel is opened by mustard or some normally compensating reduction is blocked. The fact that the oxygen uptake of the tissue diminishes after exposure to mustard makes the first alternative unlikely. Normally the tissue consumes the stroma lactate, presumably by oxidation, and reasons have been given above indicating that this utilization of stroma lactate takes place at, or near, the stroma-epithelium boundary. When lactate is oxidized something else is

reduced. Mustard inhibits the utilization of stroma lactate, consequently at some point higher up in the affected hydrogen transport system something else fails to be reduced. We have, therefore, a potential link between the metabolic effects of mustard on lactate utilization and its effects on the cohesive boundary.

Under anaerobic incubation the loosening effect by mustard does not occur, and, in addition, some repair of the primary damage does occur, for subsequent aerobic incubation results in significantly less loosening of the epithelium than aerobic incubation without preliminary anaerobic incubation. There is a similar effect of a period of anaerobiosis following exposure to mustard in respect to the inhibition of lactate utilization. Moreover, the dosage range of mustard in respect to these two effects is identical. Finally, the effect on lactate utilization develops within two hours after exposure, while the loosening requires about six hours of incubation to become manifest. All this can be brought together in the supposition that the boundary itself contains components of the hydrogen transport system for utilization of the stroma lactate, and that oxidation of some of these components destroys some of the cohesive forces, perhaps by rendering these components susceptible to proteolytic cleavage.

NUCLEAR EFFECTS

In formulating an hypothesis as to the mechanism of mustard injury which leads to a loosening of the corneal epithelium, one can at least attempt to link the pathological phenomenon with those metabolic disturbances that have been found to be associated with it. Even such a tentative guide is lacking when we face the problem of the disturbances in nuclear physiology which result from exposure to mustard, for these disturbances occur at dosage levels at which no metabolic abnormalities have as yet been found. An attempt at an hypothesis regarding these effects can be guided only by analogy. The boundary between some tissues appears to be both the seat of active metabolic interaction and to contain components that react readily with mustard. Similar metabolic interactions and similar chemical reactivity may perhaps be found at the nuclear boundary.

IV. *Comment.* In the preceding discussion we have attempted to bring together the available information on the metabolic interactions

between epithelium and stroma, the effect of mustard on these interactions, and the effect of mustard on tissue cohesion. In order to illustrate one way in which this information might be combined, a series of unsupported hypotheses have been introduced, chiefly centering around the assumption that the metabolic interaction may be located at the boundary of cohesion. There are very serious reasons for believing that any such unifying theory is untrue, and this applies, a fortiori, to the particular hypotheses that have been suggested to illustrate how a unifying theory might be constructed. Each of the phenomena that we have sought to bring together is complex, and our knowledge of each is very incomplete. Moreover, the effects of mustard are themselves complex, and several different effects have been shown to be mutually independent. If our work in this field were to be continued, we should not have the temerity to suggest these unsupported hypotheses, but should merely use them as guides in the choice of further experiments.

The justification for formulating these hypotheses at the present time, however, is not merely to pass on to others our current working hypotheses, however small their value may be, but to show that the subject matter has actually reached a stage at which specific working hypotheses and a tentative general theory can be formulated in terms susceptible to experimental verifications or rejection. The gap in our knowledge between the biochemical and the pathological effects of mustard is still unbridged, but we can at last ask specific questions as to the possible construction of some specific bridges.

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APPENDIX I

THE TOLERANCE OF RABBIT CORNEA FOR VARIOUS CHEMICAL SUBSTANCES*

WILLIAM F. HUGHES, JR.,

In the course of the studies reported in the preceding papers a great number of substances were injected into the corneas of rabbits and the resulting reactions observed. These experiments were performed, in part, as controls for a wide variety of special studies, in part as a preliminary survey of the general toxicology of the corneal tissue. The results are compiled in the table below.

Unless otherwise noted, 0.1 ml. of the solution of test substance was injected intracorneally, using a #25–27 gauge needle and tuberculin syringe. Occasionally the anterior chamber was entered accidently, in some cases resulting in a persistent edematous bulging of the cornea which could be identified clinically and the false positive corneal reaction discarded. The exact quantity injected within limits of 0.05 cc.–0.2 cc. was of less importance than the concentration of the injected material. Secondary infection was uncommon. Accidental injection of air into the cornea did not increase the severity of the reaction produced.

The reactions following intracorneal injection were similar in severity to those following mechanical removal of the corneal epithelium with a cotton toothpick swab, followed by irrigation for 10 minutes with the test solution.

A numerical grading of the severity of the ocular reaction follows the scheme outlined previously (1). The single numerical value represents the sum of the maximum values of each symptom observed over a period of 7–14 days, expressed in percentage of the maximum possible total.

Concentrations of solutions are expressed in terms of Molarity ("M") unless otherwise specified.

REFERENCES

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TABLE I

Tolerance of Rabbit Cornea for Various Chemical Substances

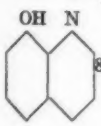
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Miscellaneous Agents (Hypertonic, Dehydrating, Agents, Solvents, etc.)				
H_2O	0		0	All hypotonic solutions of non - toxic agents.
NaCl	0.9% (0.16M)		0	Isotonic
	2.3%	1	5	Hypertonic- small bullae produced
	4.5%	1	10	Large bullae
	9.0%	1	25	Edematous bulging of cornea
C_2H_5OH	50%	1	20	Non - progressive lesion
	3%	1	0	
C_6H_5OH (Phenol)	0.08M	1	10	
C_5H_5N (Pyridine)	0.08M			
	pH8	1	0	
	pH7	1	0	
2,4 dinitrophenol	0.05M	1	52	
	0.012M	1	2	
	0.003M	1	5	
HCHO Formaldehyde	5.3M	1	70	
	0.33M	1	52	
	0.016M	1	37	
 8-hydroxy-quinoline	0.01M	1	5	
$(C_2H_5)_2 = N-C-SH$ diethyldithiocarbamate	0.05M	2	0	
	0.01M	2	0	

TABLE I—*Continued*

SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Miscellaneous Agents—Continued				
Methyl Glyoxal CHO | C=O | CH_3	1%	1	60	
	0.25%	1	10	
	0.06%	1	2	
	0.015%	1	0	
Putrescine $NH_2(CH_2)_4 \cdot NH_2$		2	16	
Taurine $NH_2(CH_2)_2SO_3H$		2	15	No corneal reaction until 4 days after injection in one eye.
Cadaverine $NH_2(CH_2)_5 \cdot NH_2$		2	1	
Solvents				
Propylene Glycol	5 min. irrigation with 100% P.G.	1	50	Hydroscopic agent
	5 min. irrigation with 50% P.G. in water	1	2	
	2 drops	10+	4	Conjunctival irritation only
	0.1 cc. intracorneally	1	25	
Ethylene Glycol	2 drops	10	4	Conjunctival irritation only
Thiodiglycol	5 min. irrigation	1	62	
	1 min. irrigation	1	25	
	2 drops	5	4	
	0.1 cc. intracorneally	4	45	

TABLE I—*Continued*

SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Solvents—Continued				
Isopropyl Alcohol	3 min. irrigation with 50% Isopropyl alcohol in water	1	45	
Aerosol	1 min. irrigation with 8% solution	1	32	
	4 min. irrigation with 1% solution	1	32	
	0.1 cc. of 1% intracorneally	1	50	
Triacetin	6 min. irrigation	1	0	Non - hydroscopic
	0.1 cc. intracorneally	1	25	
Protein - Precipitating Agents (Anions)				
Hydrochloric Acid or Trichloracetic Acid	<pH3		+	
	>pH3		0	
"Universal Buffer" (citrate-phosphate-borate)	<pH 4.5		+	
	>pH 4.5		0	
Metaphosphoric Acid or Sulfosalicylic Acid	<pH 5.5		+	
	>pH 6.0		0	
Picric acid or Tungstic acid or Tannic acid	pH 7–9		+	
Mucoid - Dissolving Agents (Alkalies)				
NaOH	>pH 11.5		+	
	<pH 11.5		0	
$Ca(OH)_2$	0.014M (Sat. Soln.)	1	52+	

TABLE I—*Continued*

SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Metal Ions				
$CaCl_2$ (neutral solution)	5.4M sat. soln.	1	75	Hypertonic
	1.0M	1	50	Hypertonic
	0.08M	6	2	Intracorneal injections
	0.08M	5	45	Mechanical removal of corneal epithelium followed by 10 min. irrigation
$BaCl_2$	0.08M	2	0	At pH5 or 7. Intracorneal
	0.08M	10	50	Mechanical removal of epithelium followed by 10 min. irrigation.
$MgSO_4$	0.08M	1	0	
$FeCl_3$	0.05M	1	32	
	0.003M	1	32	
	0.0015M	2	4	
$Pb(Ac)_2$	0.08M	2	100	Same reactions produced at pH 4.2 and pH 6.0
	0.1–0.01M	3	50	
	0.003M	2	35	
	0.0012	1	7	
	0.0006	1	0	
	0.0003	1	0	
$ZnCl_2$	0.08M	1	40	
	0.0025M	2	25	
	0.0013M	1	35	
	0.0006M	1	38	

TABLE I—*Continued*

SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Metal Ions—Continued				
$ZnCl_2$	0.0003M to 0.00004M	4	0	
$CuCl_2$	0.16M	1	72	pH 5.0
	0.08M	1	65	pH 5.5
$CuSO_4$	0.008–0.002M	3	30	
	0.001–0.0005M	3	17	
$HgCl_2$	0.08M	1	75	
	0.001M	1	60	
	0.006M	2	22	
	0.003M	2	8	
	0.00015M to 0.00002M	4	0	
Arsenicals				
ClCH=CH−$AsCl_2$ (Lewisite)	0.01Mgm		75	Calculated from analysis of arsenic intracorneally
C_6H_5AsO Phenylarsine oxide	0.01M	1	100	
Mapharsen	0.04M	1	85	
	0.004M	1	40	
$NaAsO_2$ Sodium meta-arsenite	0.01−			
	0.005M	2	87	
	0.0008M	1	22	
	0.0004M	1	15	
	0.0002M	1	5	
	0.0001M	1	0	
Na_2AsO_4 Sodium arsenate	0.08M	4	75	

TABLE I—*Continued*

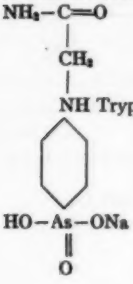
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Arsenicals—Continued				
 Tryparsamide	0.04M	1	0	
	0.004M	1	0	
Sodium selenite	0.05M	1	97	
	0.012M	1	82	
	0.003M	1	77	
Oxidizing Agents				
H_2O_2	0.3%	1	60	Intracorneally
	0.25%	1	95	Irrigation
	0.12%	1	62	
	0.10%	1	50	
	0.06%	1	50	
	0.03%	1	45	
	0.01%	1	30	
	0.003%	1	37	
	0.001%	1	0	
$KMnO_4$	0.08M	1	60	Intracorneally
	0.028M	1	35	Irrigation
	0.009M	1	50	
	0.003M	1	0	
	0.001M	1	0	
$K_3Fe(CN)_6$	0.07M	2	5	
I_2 in 3% KI	0.1M	1	95	
	0.02M	2	30	
	0.01M	1	15	
	0.005M	1	0	
KI alone	3%	1	17	
	1%	1	0	
KIO_3	0.16M	1	22	
	0.03M	2	0	pH 5 and pH 7.0

TABLE I—*Continued*

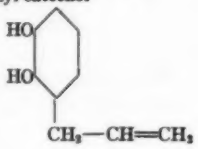
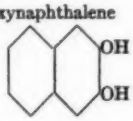
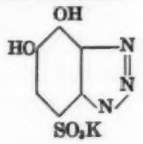
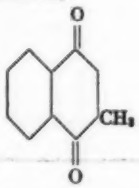
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Oxidizing Agents—Continued				
$NaClO_4$ (Perchlorate)	0.08M	2	3	
$NaClO_3$ (Chlorate)	0.08M	1	20	
$Na_2Cr_2O_7$ (dichromate)	0.08M	1	70	
Na_2CrO_4 (chromate)	0.08M	1	75	
Quinones and Hydroquinones				
3-Allyl-catechol 	0.05M	1	45	
	0.025M	1	12	
	0.012M to 0.0015M	4	0	
Dihydroxynaphthalene 	0.05M	2	42	
Potassium - benzotriazole - hydroquinone sulfonate 	0.05M	2	37	
2 Methyl 1,4 naphthoquinone (Vitamine K) 	0.05M	2	38	
	0.025M	4	18	

TABLE I—*Continued*

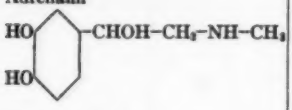
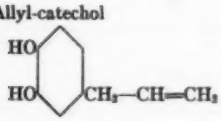
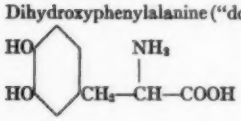
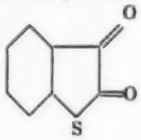
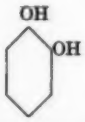
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Quinones and Hydroquinones—Continued				
Adrenalin 	0.05M 0.025M	1 2	17 12	
Vit. K_+ Adrenalin	0.025M of each	2	35	
4-Allyl-catechol 	0.05M 0.025M 0.012–0.003M 0.015M	1 1 3 1	37 30 5 0	
Dihydroxyphenylalanine ("dopa") 	0.05M	2	22	
Thionaphthenequinone 	0.05M	2	12	
p-hydroquinone	0.05–0.012M	3	5	
catechol 	0.05–0.012M	3	0	

TABLE 1—*Continued*

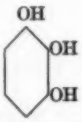
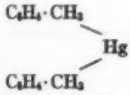
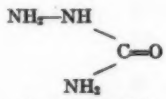
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Reducing Agents				
$Na_2S_2O_3$ (thiosulfate or "hypo-sulfite")	0.08M	2	15	
	0.02–0.01M	2	2	
	0.008–0.005M	3	5	
	0.0025–0.0006M	3	2	
$NaHSO_3$ (bisulfate)	0.08M	1	7	
	0.008M	1	0	
$NaNO_2$ (nitrite)	0.08M	1	0	
 (Pyrogallol)	0.08M	1	10	
	0.008M	1	7	
Sulfhydril-Binding Agents				
ICH_2COOH (Iodoacetate)	0.1M	1	100	
	0.001M	1	70	
$C_6H_5 \cdot HgOH$ (Phenyl-mercuric hydroxide)	0.1M	1	77	
	0.001M	1	25	
$C_6H_5 \cdot HgCl$	0.1M	4	66	
	0.01M	1	47	
	0.001M	1	37	
	0.01M	1	0	Control, does not bind sulfhydril groups
	0.001M	1	0	
Miscellaneous Inhibiting Agents				
NaCN (cyanide)	0.1M	3	46	
	0.001M	1	10	
NaN_3 (azide)	0.1M	3	2	pH 6 and pH 7
	0.001M	1	5	
 (semicarbazide)	0.1M	1	0	
	0.001M	1	0	

TABLE I—*Continued*

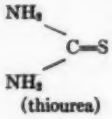
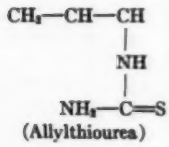
SUBSTANCE	CONCENTRATION	NO. EYES TESTED	SEVERITY OF REACTION	NOTES
Miscellaneous Inhibiting Agents —Continued				
 (thiourea)	0.08M	1	22	Late reaction
 (Allylthiourea)	0.05M	2	18	
	0.01M	2	6	
NaF (fluoride)	0.02M	1	25	
	0.01M	1	15	
	0.005–0.006M	4	0	
Histamine	1:10,000	2	0	Moderate conjunctival edema up to 3 hours after injection
	1:100,000	1	0	
	1:1,000,000	1	0	
Pontocaine	0.5%	1	42	
Eserine	0.25%	1	2	
Dyes				
Crystal Violet (phenylmethene Rosaniline dye)	0.5%	1	57	
	0.06%	1	70	
	0.007	1	15	
Phenosafranin (azin dye)	0.5%	1	67	
	0.06%	1	70	
	0.007%	1	0	
Acriflavine (xanthene - acridine dye)	0.1%	2	42	pH 7.0 or pH 3.5
	0.05%	1	45	
	0.01%	1	45	
Brom Phenol Blue (xanthene-sulphonphthalein dye)	0.05%	1	32	

TABLE I—*Concluded*

SURFACE	CONCENTRATION	NUMBER OF EYES TESTED	SEVERITY OF REACTION	NOTES
Methylene Blue (Thiazine dye)	0.5%	1	35	
	0.06%	1	37	
	0.007%	1	0	
Malachite Green (phenylmethane dye)	0.05%	1	35	
Toluidine Blue (thiazine dye)	0.5%	2	28	
	0.1%	1	7	
	0.05%	2	11	
Methyl Green (Rosanilin dye)	0.05%	1	15	
Fluorescein (xanthene dye)	0.05%	1	6	
Congo Red (Azo dye)	0.5%	1	9	
	0.06%	1	12	
	0.007%	1	0	
Pyronin (xanthene dye)	0.05%	1	0	
Thionine (thiazin dye)	0.05%	1	0	
Rose Bengale (xanthene dye)	0.05%	1	0	

APPENDIX II

A MECHANICAL DEVICE FOR THE EXTRACTION OF SOLUBLE COMPOUNDS FROM THE CORNEA AND OTHER TOUGH TISSUES*

JONAS S. FRIEDENWALD AND SYLVIA G. MOSES

Biochemical studies on the cornea, skin, and other tough tissues are often handicapped by the amount of manual labor involved in grinding the tissue. Thus, in determining the acid soluble, non-protein nitrogen of the cornea, it was necessary to grind each sample in a mortar with the extracting fluid for 15 minutes. None of the available tissue grinders or homogenizers (1) are suitable for extraction of such tissues. However, the mechanical device illustrated in the figures was found to yield equally satisfactory extracts. It consists of a vessel (Figs. 1, 2) at the bottom of which is a pit suitable to contain the tissue sample which is to be extracted. A plunger, the tip of which fits loosely into this pit, is used to squeeze the tissue. Above the tip of the plunger is a flange which fits loosely into the main cylindrical compartment of the vessel and prevents the extracting fluid from splashing when the plunger is lowered. Three grooves are cut in the margin of the flange to allow air or fluid to pass the flange as the plunger is raised or lowered. The plunger is moved slowly up and down by a lever. With a lever ratio of 10, a weight of 2 lbs. on the end of the lever was found convenient, and 200 strokes of the weighted lever sufficient to produce as complete an extraction as 15 minutes grinding in a mortar, in each case with two washings of the tissue.

If single samples are to be extracted, the lever may be operated manually. For larger scale operation, six vessels were mounted in a frame, each under its plunger and lever. The plungers were raised and lowered by a set of eccentric wheels attached to a shaft turned at a rate of one revolution every 4 seconds by a $\frac{1}{50}$ HP motor with an appropriate reduction gear.

* The work described in this paper was done in largest part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Johns Hopkins University.

Our use of this device has been concerned chiefly with non-protein nitrogen determination in trichloracetic acid extraction. For this purpose vessels and plungers were machined from a 2 inch Lucite rod. The

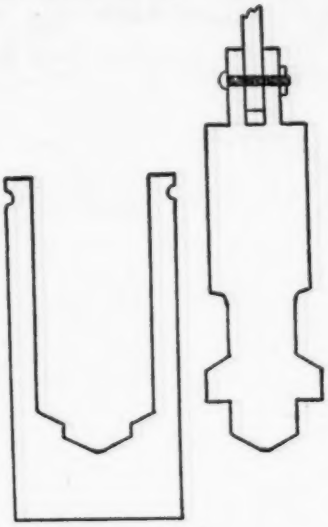


FIG. 1

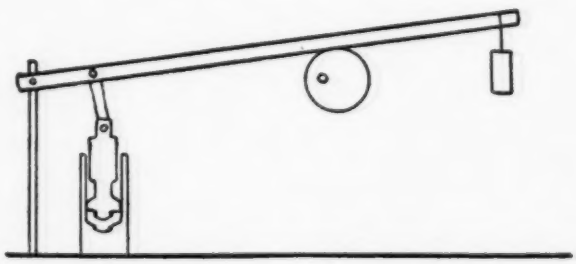


FIG. 2

procedure arbitrarily adopted for convenience in using the mechanical extractor consists of a ten minute extraction followed by two five minute washings. After using this device for several hundred samples, we have found it completely satisfactory. It might be mentioned that

we were surprised to find that the simple process of squeezing in this manner gave as good results as the squeezing plus shearing of manual manipulation in a mortar.

REFERENCES

1) UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F.: Manometric Technique and Related Methods for the Study of Tissue Metabolism. Burgess Publ. Co., Minn. 1945.

BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this Bulletin)

Nurse-Patient Relationships in Psychiatry. By HELENA WILLIS RENDER. Illus. 346 pp. $3.00. *McGraw-Hill Book Company, Inc., New York, New York* 1947.

Mrs. Render has made an outstanding contribution to the field of psychiatric nursing. Her book is instructive and direct, yet detailed and lucid. Her suggestions come from a keen and warm understanding of the needs of patients and are practical and sound. She has shown the field of psychiatric nursing to be worthy of the sincere efforts of a mature and understanding person, and has revealed the dignity, satisfaction, and challenge in this field of nursing. This is an excellent book and might be read with profit by both doctors and nurses.

A. B.

Nursing Care in Chronic Diseases. By EDITH L. MARSH. Illus. 237 pp. $3.25. *J. B. Lippincott Company, Philadelphia, Pennsylvania,* 1946.

The objective of this book, as stated in the foreword, is "to promote for the greatly neglected chronic invalid a high degree of enlightened care and human understanding."

The subject matter includes a review of home and hospital facilities available for the chronically ill and a frank, unbiased discussion of present day inadequacies in care. As the various chronic conditions are presented for study, one is impressed by Miss Marsh's broad sociological viewpoint and her unmistakable interest in the patient as a sick person in need of more and better nursing care. She emphasizes the value of cheerful, sympathetic, humane care, and of treating each patient as a rehabilitation problem even though the result may be only of a temporary nature. Miss Marsh has made this a truly inspirational and practical book which should be helpful to students and faculties in schools of nursing and to the staffs of social and public health agencies.

V. B.

Professional Adjustments. By SISTER MARY ISIDORE LENNON. 299 pp. $3.00. *The C. V. Mosby Company, St. Louis, Missouri,* 1946.

In this book Sister Mary Isidore Lennon has endeavored to set forth in simple, comprehensive form material which should enable the beginning student in a school of nursing to achieve happiness in her service of the sick, in her studies, and in her social life. She writes with a real understanding of the difficulties confronting the very young nursing student of today who, in most of our hospitals, is expected overnight to cast aside the carefree ways of adolescence and to acquire the maturity which should characterize the professional woman.

The author writes primarily for students in Catholic hospitals and emphasizes the Catholic viewpoint, which limits somewhat the usefulness of the book. It probably would not be acceptable as a text for non-Catholic students, although the practical suggestions offered along the lines of character development and ethical practice in nursing would undoubedly be of interest to lay instructors in schools of nursing.

V. B.

Retinal Structure and Colour Vision. By E. N. WILLMER. Illus. 431 pp. $4.50. *The Macmillan Company, New York, New York,* 1947.

Under the new assumption that the rods, comprising 95% of the visual receptors, are not blind in daylight, the author boldly discusses the possibility that rods and cones contribute equally to the effects of color mixture: rods giving blue-greens and cones red-yellow. Forced, however, by the evidence, to three variables, he postulates the "day-rod", whose resultant color sensitivity is that of rhodopsin minus iodopsin, achieved by neural inhibition. Poliak provides a structural basis for this.

Present data, at the level of bipolar and ganglion cells, insufficiently support the author's attempt to explain by this hypothesis the usual test cases of color phenomena. Consequently his quantitative discussion is quite unconvincing. Adaptation and brightness are particularly unsatisfactory. His main contribution is the demonstration that known neural behavior suffices qualitatively to account for chromatic phenomena, without postulating the unobserved photopigments in cones required by current color theories. The use of recent data from Granit, Hartline and Poliak lends the argument a force and verisimilitude denied to E. Q. Adams' more adequate theory of bipolar inhibition (1924). Willmer's striking observations on the blue-blindness of the fovea are still controversial quantitatively; so that the assignment of blue response to rod function, though now possible on grounds of retinal structure, is yet unproved.

Excellent illustrations, several in color, lend interest to a rather technical work, intended for readers familiar with the complexities of color vision.

S. A. T.

Principles and Practice of Medicine, The. (Originally written by William Osler.) By HENRY A. CHRISTIAN. 16th ed. 1539 pp. $10.00. D. Appleton-Century Company, Inc., New York, New York, 1947.

The original text of this book was written by Osler and published in 1892. During the past nine years, under the editorship of Henry A. Christian, it has undergone radical changes and modernization. There is still much of the original manuscript, but one is frequently aware of the present author's literary style.

In this edition, Dr. James G. Carr, in a short preliminary paragraph, traces the history of medicine (1892–1947) as told in sixteen editions of Osler's "Practice and Principles of Medicine." It is really quite interesting to follow the changes in the importance of various diseases, the emphasis on physiological abnormality instead of gross and microscopic pathology, and the revolutionary change from therapeutic

nihilism to the profuse renaissance of present day therapeutic enthusiasm. It is heartening to consider the many advances in the principles and practice of medicine that these sixteen editions of Osler's textbook record.

In this edition, Dr. Christian genuflects very slightly to psychosomatic medicine. He points out:

"In latter years, with the enormous development of laboratory procedures and the increased utilization of complex techniques of examining the patient, the physician has found it increasingly easy to neglect the patient's personality and surroundings in his management of him, with a resultant feeling on the part of the patient that he is missing something that is traditional of the general practitioner that served his forebearers. As a resultant, renewed emphasis is coming to be placed again on the emotional and functional aspects of disease, with an increased introduction into our hospitals of psychoneurologists to turn the minds of the students and internes toward functional disease and functional aspects of organic disease in an effort, as yet not too satisfactory, to rebridge a recognized dilemma in modern medicine with a point of view expressed in the terms, Psychosomatic Disease and Psychosomatic Medicine. Often this is looked upon as something new. Its newness, however, chiefly is in the terms now used for what the former generations of medical men practiced without much use of names for it."

This edition, however, opens "with a discussion of some of the diseased conditions now recognized as essentially non-organic in nature, in the hope that from reading a discussion of them the student and practitioner will obtain knowledge toward a more satisfactory understanding of what ordinarily is looked upon as purely organic disease. It is to be recognized that every patient with organic disease presents a functional, emotional reaction which varies with the personality, the economic and social relationship, and the race and heredity of the individual, and that his problem as a patient can be solved only by integrating his functional and somatic disabilities after a thorough study of both."

The book is up to date in its detailed description of penicillin therapy, of streptomycin therapy, of the part that sulfonamides play. One is interested to see how in a book of some 1374 pages endocrine diseases are accounted for in 40 pages while the same space is devoted to pneumonia, and typhoid fever fills 30 pages. A feature which Dr. Christian introduced several editions ago is the "References" at the end of each chapter. In the references are usually one or two of the early classic papers and a few of the outstanding recent ones. This allows the student to pursue any subject further.

Some of us who became very well acquainted with tropical diseases may regret that Dr. Christian has not made more of an effort to bring these subjects up to date. However, one must confess that we see very few of these diseases that our armies have brought back with them from the tropics.

This is an excellent book. Dr. Christian has performed a brilliant task, and those of us with a sentimental feeling for the old Osler "Textbook" owe him a debt of gratitude.

H. M. T., Jr.

BOOKS RECEIVED FOR REVIEW

Calcific Disease of the Aortic Valve. By HOWARD T. KARSNER and SIMON KOLETSKY. Illus. 111 pp. $5.00. *J. B. Lippincott Company, Philadelphia, Pennsylvania*, 1947.

Diabetes and the Diabetic in the Community. By MARY E. TAGNEY. Illus. 259 pp. $2.75. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Endogeneous Endocrinotherapy, Including the Causal Cure of Cancer Compendium. By J. SAMUELS. Illus. 539 pp. *Holdert & Company, Amsterdam, Holland*, 1947.

Essentials of Pharmacology. By FRANCES K. OLDHAM, F. E. KELSEY, and E. M. K. GEILING. Illus. 440 pp. $5.00. *J. B. Lippincott Company, Philadelphia, Pennsylvania*, 1947.

Essentials of Prescription Writing. By CARY EGGLESTON, 8th Edition. Illus. 155 pp. $2.00. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Fundamentals of Neurology. By ERNEST GARDNER. Illus. 336 pp. $4.75. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Fundamentals of Psychiatry. By EDWARD A. STRECKER. 325 pp. $4.00. *J. B. Lippincott Company, Philadelphia, Pennsylvania*, 1947.

Gifford's Textbook of Ophthalmology, 4th Edition. By FRANCIS H. ADLER. Illus. 512 pp. $6.00. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Hodgkin's Disease and Allied Disorders. By HENRY JACKSON, JR. and FREDERIC PARKER, JR. Illus. 177 pp. $6.50. *Oxford University Press, New York, New York*, 1947.

Pharmacology, Therapeutics and Prescription Writing. By WALTER A. BASTEDO. Illus. 840 pp. $8.50. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Plague: Laennec (1782–1826), Inventor of the Stethescope and Father of Modern Medicine. By ARTHUR N. FOXE. Illus. 122 pp. $2.50. *Hobson Book Press, New York, New York*, 1947.

Surgery of the Ambulatory Patient. By L. KRAEER FERGUSON. Illus. 932 pp. $10.00. *J. B. Lippincott Company, Philadelphia, Pennsylvania*, 1947.

Textbook of Bacteriology. By THURMAN B. RICE. Illus. 603 pp. $6.50. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Textbook of Clinical Neurology. 6th Ed. By ISRAEL S. WECHSLER. Illus. 829 pp. $8.50. *W. B. Saunders Company, Philadelphia, Pennsylvania*, 1947.

Textbook of the Nervous System. By H. CHANDLER ELLIOTT. Illus. 384 pp. $8.00. *J. B. Lippincott Company, Philadelphia, Pennsylvania*, 1947.

Trichomonas Vaginalis and Trichomoniasis. By RAY E. TRUSSELL. 277 pp. $6.00. *Charles C. Thomas, Springfield, Illinois*, 1947.